Molecular Characterization of Drug-Resistant *Mycobacterium tuberculosis* and Evaluation of the Performance of GeneXpert MTB/RIF® Assay using Urine Specimen to Diagnose Pulmonary Tuberculosis in Sputum-scarce Patients in Addis Ababa, Ethiopia

A dissertation submitted to the Addis Ababa University College of Natural Science Department of Microbial, Cellular and Molecular Biology for partial fulfillment of the requirements for Degree of Doctor of Philosophy (PhD) in Biology (Applied Genetics)

By

Eyob Abera Mesfin

Addis Ababa University
Addis Ababa, Ethiopia
June 2018

DECLARATION

I, the undersigned, declare that the dissertation hereby submitted for the Degree of Doctor of Philosophy (PhD) to the Addis Ababa University is my own work and has not previously been submitted at another university. The materials obtained from other sources have been duly acknowledged.

Name: Eyob Abera Mesfin **Signature:**_____ **Date:**_____

I. Acknowledgments

Praise be to God, the most gracious, and the most merciful who has made everything happen. I express my appreciation and gratitude to my supervisors, Dr. Dereje Beyene (AAU), Dr. Belay Tessema (University of Gondar), and Dr. Abraham Tesfaye (Addis Ababa Health Bureau Health) for their consistent guidance and invaluable suggestions and comments throughout the thesis work. Your scientific advice, generous support and encouragement gave me confidence to think critically and to work independently. I found them so optimist, helpful, polite, consistent and stable in all interactions we have had so far. Without their significant contribution, it would have been difficult to accomplish this work.

I am grateful to Ethiopian Public Health Institute (EPHI) for allowing me to do laboratory tests for culture, and DST and Molecular and Experimental Mycobacteriology Research Center Borstel, Germany for molecular experimental part of this study. Moreover, I would like to acknowledge with gratitude IFCC for financial support during my study in Germany and also Ethiopian Medical Laboratory Association for facilitating the financial support. I extend my thanks to AAU, DMCMB for some financial contribution to my thesis work. In this regard, I am grateful to Dr. Gurja Belay for facilitating administrative and financial support.

I would also like to thank Zelalem Yaregal head of EPHI National TB Reference Laboratory for giving me the opportunity to perform analysis and Mr. Desalegn Addise for his professional guidance and support during the laboratory analysis and all staff: Abebaw Kebede, Miskir Amare, Biniyam Dagne, Ephrem Tesfaye, Getu Diriba, Bazezew Yenew, Mengistu Tadese, Hilina Molalegn, Aynalem Alemu, Waganeh Sinishaw, Abyot Meaza, Shewki Moga, Muluwork Getahun, Sinatayehu Gebeyehu, Aberash Belete and other staffs for their support and guidance during my laboratory analysis.

I would like to thank Prof. Stefan Niemann, for giving me the opportunity to perform the genotyping analysis at Molecular Mycobacteriology Research Centre, Borstel, Germany and Dr. Matthias Merker who gave me the unique opportunity to work in the laboratory with his guidance and assistance during analysis of genotyping data using bionemerics software. I would also to thank Anja Lüdemann and Tanja Struve for their assistance during genotyping analysis, and Yassir Adam for his assistance in analysis of genotyping data, and Ms. OLA S. for her assistance during sequencing, and their hospitality and sharing experiences.

I would like to express my heartfelt gratitude to Dr. William L. R. Emmet for his advice and editing the thesis, Mr. Anteneh Tessema for his guidance in the statistical analysis and Ms. Adanu Zewdu for her assistance on the editing of the thesis. I would like also to thanks Dr. Aster Shewamare and Dr. Addisu Admasu for their guidance and advice in the clinical aspect of the study. I would like to acknowledge the study subjects, data collectors, clinicians and lab staff from study sites, Addis Ababa Health Research and Laboratory Services staffs for their technical assistance.

I have no words powerful enough to explain my mother Silas Fikadu, for her unconditional and truly love and dedication at all times. I am deeply indebted to my father in heaven, for raising me with love, and care with that short period of his life. So I would like to dedicate this thesis to my mother and father. My brothers, my sisters and all family members: Solomon Abera, Tseahye Binega, Zufan Binega, Meaza Binega, Eden Binega, Azeb Binega, Meron Delelegn, Daniel Taddese and Fiker Tsehaye thank you very much for your sincere love, concern, care and support throughout my life. I gratefully appreciate Roza-Beti for your care, love, and courage to realize my study. My sincerely appreciation and gratitude go to my friends and fellow colleagues who in one way or the other significantly encouraged and supported me towards the end of my study: namely Mesfin Asfaw, Eshetu Lemma, Addis Zewde, Daniel Akuma, and Tewodros Girmay.

Finally, I would like thank again Almighty God, for giving me a favor in your sight. Thank you!

II. Table of Contents

Contents **Page**

III. List of Table

IV. List of Figures

V. Abstract

Multidrug drug-resistant tuberculosis (MDR-TB) is a major health problem and seriously threatens TB control globally. Ethiopia is among the 30th highest TB burden countries. MDR-TB is a result of unsuccessful TB control programs and associated with mutations in drug target genes. Another challenge of TB is that about one-third of TB/HIV co-infected patients fail to produce a sputum specimen for diagnosis. Understanding of the pattern of resistance and genotyping of strains, and identify alternative specimen for diagnosis are important for TB/MDR-TB control. Thus, the aim of this study was to determine the pattern of mutations associated with resistance, genotype, and performance of GeneXpert MTB/RIF using urine specimens. A cross-sectional study was conducted in Addis Ababa from June 2015 to December 2016. Sputum specimens and socio-demographic data were collected from 358 MDR-TB suspected cases, and 150 urine specimens from sputum scarce cases and cases capable of giving sputum suspected of pulmonary TB. Specimens were analyzed using Ziehl-Neelsen, GeneXpert MTB/RIF assay, and cultures. All culture-positive specimens were analyzed for drug susceptibility tests. Sequence analysis for rpoB, katG, fabG1-inhA, embB, pncA, rrs and rpsL genes, and genotyping was performed using 24 MIRU-VNTR and spoligotype. Data were analyzed using SPSS version 23.

Of 358 MDR-TB suspected cases, 226 were culture positive for *Mycobacterium tuberculosis,* of these, 162 (71.7%) had been previously treated for tuberculosis, while 128 (56.6%) were TB/HIV co-infected. An analysis of drug resistance indicated that 110 (48.7%) strains were resistant to isoniazid, 94 (41.6%) to streptomycin, 89 (39.4%) to rifampicin, 72 (31.9%) to ethambutol, and 70 (30.9%) to pyrazinamide. Among the 226 study participants, 89 (39.4%) were determined to be MDR-TB with TB/HIV co-infection, cigarette smoking, alcohol drinking, and admissions and/or visits to hospitals significantly ($p<0.044$) associated with the observed MDR-TB prevalence. Among the 209 isolates submitted for drug target gene sequencing, 101 (48.3%) isolates had the mutations in a katG gene, of these, 98 (98.0%) mutations were found at codon S315T, and other

mutations were found 90 (43.1%) isolates in rpoB gene. Out of these, 61 (67.8%) and 15 (16.7%) mutations occurred at codons S531L/W and H526Y/S/D/L respectively. Moreover, 73 (34.9%) isolates had a mutation in embB gene, and majority 48 (65.8%) of the mutations were found at codon M306I/V/L. In another finding, 68 (30.1%) isolates had a mutation in pncA gene with high mutation at codon 65 in 19 (27.9%) isolates. Likewise, mutations in rrs and rpsl genes were found in 56 (26.8%) isolates and the highest 50 (89.3%) proportion of mutation was found in rpsL gene at codon K88R/T and K43R. Furthermore, the least frequency of mutation was observed in fabG1-inhA promoter region in 7 (3.3%) isolates.

A total of 167 isolates had a valid genotyping results, of thse 157 (94.0%) were classified as known lineages; Dehli/CAS 50 (29.9%), TUR 44 (26.3%), H37Rv like 25 (15.0%), TUR_Ethiopia_3 17 (10.2%), Haarlem 14 (8.4%), Ural 4 (2.4%), LAM 1 (0.6%), X-type 1 (0.6%), and EIA 1 (0.6%). The remaining 10 (6.0%) isolates were undefined. Among MDR-TB, the highest predominant genotype was TUR with 35 (46.1%). Cluster analysis showed that a total of 103 (61.7%) strains shared a genotyping pattern, and a recent transmission index was 50.3%. In another finding, of the 150 urine specimens tested, 5 (3.3%) were positive by GeneXpert MTB/RIF and culture. The sensitivity and specificity of GeneXpert MTB/RIF were 100% and 99.3% (95%CI: 97.4-100%) respectively, and overall performance agreement was 99.3% (95% CI: 97.4-100%).

In conclusion, a highly diverse *M. tuberculosis* population structure was found, with a predominance of the Dehli/CAS genotype. The prevalence of MDR-TB in the study population was significantly high and the predominant lineage among MDR-TB strains was TUR, and there was a high rate of recent transmission among MDR-TB strains. A substantial number of mutations were observed at codons 531, 315, 306, 65, and 88 in rpoB, katG, embB, pncA, and rpsl genes respectively. TB/HIV co-infection, smoking of a cigarette, alcohol drinking, admission and visit of health facility were identified as risk factors for developing MDR-TB. A good sensitivity and specificity of GeneXpert MTB/RIF were observed using urine specimen, thus, urine can be used

as an alternative specimen for diagnosis of PTB from sputum scare patients. In general, our findings support us to conclude that there is weak health system for prevention and control of MDR-TB/TB in Ethiopia. Therefore, effective strategies should be designed considering the identified risk factors for control of MDR-TB.

Keywords: *Mycobacterium tuberculosis*, MDR-TB, Risk factors rpoB, katG, embB, pncA, rpsL, Mutation, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide, Streptomycin, GeneXpert MTB/RIF, Urine, Sensitivity, Specificity, Genotyping.

VI. Abbreviations and Acronyms

AFB	Acid Fast Bacilli
AAU	AAU Addis Ababa University,
DMCMB	Department of Microbial, Cellular and Molecular Biology
AIDS	Acquired immunodeficiency syndrome
AM	Aminoglycosides
AMK	Amikacin
AST	Antibiotic Susceptibility Testing
BCG	Bacillus Calmette-Guérin
CSA	Central Statistical Agency
CDC	Centres for Disease Conotrol and Prevention
CPM	Capreomycin
CS	Cycloserine
DOT	Directly Observed Therapy-Short Course
DR-TB	Drug-Resistant Tuberculosis
DST	Drug Susceptibility Testing
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number tandem repeats
ECDC	European Centres for Disease Conotrol and Prevention
EMB	Ethambutol
EPTB	Extrapulmonary tuberculosis
ETH	Ethionamide
FQs	Fluoroquinolones
FMOH	Federal Ministry of Health
HGDI	Hunter-Gaston discriminatory index
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KM	Kanamycin
LFX	Levofloxacin
LJ	Lowenstein-Jensen
MDR-TB	Multidrug-resistant tuberculosis
MGIT	Mycobacterial growth indicator tube
MST	Minimum spanning tree
MTB	Mycobacterium tuberculosis

MTBC	Mycobacterium tuberculosis complex
MXF	Moxifloxacin
NALC	N-acetyl- L-cystine
NTM	Non tuberculosis mycobacteria
OFX	Ofloxacin
PAS	P-aminosalisylic acid
PCR	Polymerase chain reaction
PTB	Pulmonary tuberculosis
PZA	Pyrazinamide
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
rpoB	Gene encoding for the ß-subunit of the DNA-dependent RNA polymerase
RRDR	Rifampicin Resistance Determining Region
RTI	Recent Transmission Index
SNNP	Southern Nations, Nationalities and People
STR	Streptomycin
TB	Tuberculosis
UPGMA	Unweighted pair group method with arithmetic averages
WHO	World Health Organization
XDR-TB	Extensively Drug- resistant Tuberculosis
ZN	Ziehl-Neelsen

Chapter 1

General Introduction

1. General Introduction

1.1. *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis is a genus of M. and it is a causative agent of tuberculosis (TB) disease. Mycobacteria is divided into various groups such as the *M. tuberculosis* complex (MTBC) that can cause TB, *M. leprae* that causes leprosy; and nontuberculous mycobacteria (NTM) includes all the other mycobacteria. NTMs are widely distributed in the environment and are in most cases nonpathogenic for humans. Apart from the MTC and *M. leprae*, the genus *M.* comprises more than 140 species (van Ingen, 2013), of which approximately 50 are currently considered to be etiological agents of human disease (Wagner and Young, 2004). *M. avium* and *M. intracellulare* are a common NTM species primarily affecting immunocompromised individuals (Weiss and Glassrot, 2012).

The MTBC consists of closely related species of *M. tuberculosis, M. africanum* and *M. canettii and the animal adapted species such as M. bovis* (bovine), *M. caprae* (goats), *M. pinnipedii* (seals), *and M. microti* (rodents) (Huard *et al.,* 2006). Although about 98% of human tuberculosis (TB) cases are caused by *M. tuberculosis* globally, *M. africanum* and *M. bovis* remain public health important agents of TB disease in some countries. Globally, *M.* bovis causes about 2% of human TB, moreover, *M. bovis* differs from *M. tuberculosis* by being niacin and nitrate negative, and inherently pyrazinamide resistant (Pfyffer and Palicova, 2011).

M. tuberculosis is a non-spore-forming, non-motile, and an aerobic bacterium with rod-shaped, as well as it is classified as acid-fast bacilli and has a complex cell wall structure essential for survival (Porth, 2002). The MTB cell wall is containing mycolic acids and they are covalently attached to the underlying peptidoglycan bound polysaccharide arabinogalactan, serving as a barrier. The barrier is also important for any unusual physiological characteristics of *M. tuberculosis, such a* host defense mechanisms and resistance to antibiotics (Lee *et al.,* 2005). *M. tuberculosis* grows slowly in culture, requiring an average of 2 to 3 weeks of incubation before

growth can be detected. Colonies on solid media are off-white or buff in color, with a rough appearance. The cellular morphology of TB bacilli grown in culture demonstrates cording or ropy clumps (Pfyffer and Palicova, 2011).

The first *M. tuberculosis* genome sequence was first completed as a genome of *M. tuberculosis* strain H37Rv, and published in 1998. The genome has a high guanine (G) and cytosine (C) content (65%), and comprises 4,411,532 base pairs (bp). The last re-annotation identified approximately 4,000 genes (Camus *et al.,* 2002). Mobile genetic elements such as plasmids have been reported in some mycobacterial species, but not in *M. tuberculosis*. Nonetheless, insertion sequences (IS), and other repetitive elements have been characterized in many mycobacterial species, including *M. tuberculosis*. Special attention was paid to the clustered regularly interspaced short palindromic repeat (CRISPR) sequences and IS6110 for molecular epidemiology (Wiedenheft *et al.,* 2012). The CRISPR system is used for spoligotyping, and IS6110 for restriction fragment length polymorphism analysis.

1.2. Transmission and pathogenesis of *M. tuberculosis*

TB is an infectious disease caused by the bacillus *M. tuberculosis*. It typically affects the lungs (pulmonary TB) but can also affect other sites (extra-pulmonary TB) such as the brain, pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges and any part of the body. *M. tuberculosis* is carried in airborne particles, and spread by small airborne droplets created while a person with pulmonary TB (PTB) is sneezing, coughing, talking, or singing. The droplets are very small and up to 5μm in diameter in size, which helps to pass into the lower respiratory tract and can remain suspended in the air for several hours (Riley *et al.,* 1959). Droplets of a larger size are excluded from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract (Diamond *et al.,* 1991). The transmission of *M. tuberculosis* from a TB patient to a contact person depends on exposure duration, an intensity of exposure, cough and sputum-related host factors and *M. tuberculosis* strain-related virulence

characteristics (Dheda *et al.,* 2010). However, the major source of *M. tuberculosis* infection is a person with smear-positive PTB (WHO, 2005).

Pulmonary TB is a chronic and slowly progressive disease and most of the patients usually have signs and symptoms such as a cough, weight loss, fever, night sweats and chest pain and dyspnea (Pfyffer and Palicova, 2011). Infection occurs when a person inhales droplet nuclei containing tubercle bacilli that reach the alveoli of the lungs. These tubercle bacilli are ingested by alveolar phagocytic cells and most bacilli are killed or inhibited. However, a small number of bacilli may multiply intracellularly and are released when the macrophages die and progress to form small caseous lesions. Once these lesions have developed, in patients with normal cell-mediated immunity activated macrophages may exert microbicidal activity. The bacilli may spread by way of lymphatic channels or through the bloodstream to more distant tissues and organs (for instance, lymph nodes, kidneys, brain, and bone). This process of dissemination primes the immune system for a systemic response. Most of the infected individuals will develop a cell-mediated immune response within 2–8 weeks after infection that prevents further multiplication of the tubercle bacilli. The activated T lymphocytes, macrophages, and other immune cells form a barrier shell, called a granuloma that keeps the bacilli contained and limiting further replication and spreading (Dannenberg *et al.,* 1994)

As result, most of the bacilli are killed in the granulomas, and disease progression is prevented. However, all bacilli are not completely destroyed in some individuals as *M. tuberculosis* escape killing by blunting the microbicidal mechanisms of phagocytic cells (such as phagosome-lysosome fusion, production of nitric oxide, and other reactive nitrogen intermediates). As a result, approximately 10% of cases develop active disease (primary tuberculosis), while in approximately 90% of patients the disease is controlled as a latent TB infection; TB bacilli may remain viable within dormant lesions over many years and only approximately 10% of individuals will develop the disease when the host immune system weakens in their life. However, in HIV patients this

risk will increases to approximately 10% annually. Progressive disease is characterized by weight loss, cavitations, and fibrosis. Some of the cavities eventually open into the bronchi, which allow the bacilli to spread through the airways to other parts of the lung and the outside environment during coughing (Caminero *et al.,* 2004).

TB can infect any site of the body but the site of infection is an important factor influencing clinical expression of TB (Kubica *et al.,* 1975). In fact, about 85% of TB cases are limited to the lungs, with the remaining 15% involving extrapulmonary sites, or both pulmonary and extrapulmonary sites (Farer *et al.,* 1979). This proportional distribution is substantially different among patients co-infected with *M. tuberculosis* and HIV, where extrapulmonary involvement is much more common. This is typical of what is seen in individuals with a compromised immune system, who have limited ability to contain the infection with *M. tuberculosis*, and as a consequence, hematogenous dissemination may occur with subsequent involvement of single or multiple extrapulmonary sites. Infants and the elderly are also more prone to develop disseminated disease, also referred to as miliary TB. Meningitis is the most frequent form of central nervous system TB with a peak incidence is in children younger than 4 years of age (Cole *et al.,* 2005).

1.3. The global burden of tuberculosis

About one-third of the human population can be demonstrated to have immunologic evidence of current or past infection with *M tuberculosis* (WHO, 2013a). Overall, an estimated 1.7 billion people infected with *M.* tuberculosis but about 15% will develop TB disease during their lifetime. However, the probability of developing TB disease is much higher among people infected with HIV, and also higher among people affected by risk factors such as under-nutrition, diabetes, smoking and alcohol consumption. If patients are not getting treatment, the mortality rate is high. Studies of the natural history of TB disease in the absence of treatment found that about 70% of individuals with sputum smear-positive pulmonary TB and about 20% of among smear-negative

of people with culture-positive pulmonary TB died within 10 years of being diagnosed (WHO, 2017).

The globally TB deaths and incidence rate among HIV negative patients have fallen in number since 2000 and TB deaths were decreased from 1.8 million in 2000 to 1.4 million in between 2001 and 2015. But, the TB incidence rate was decreased by 1.5% from 2014 to 2015. Moreover, globally, TB is one of the top 10 killer death and caused more deaths than HIV in 2015, and there were an estimated 10.4 million incident TB cases with 90% of adults, and 6 countries accounted for 60% of the global total such as India, Indonesia, China, Nigeria, Pakistan, and South Africa. In addition, about 11% of incident TB cases were HIV positive and more than 50% cases was fond in WHO African Region countries. In addition, there were 0.4 million deaths among HIV-positive people in 2015. (WHO, 2016). Similarly, TB continues as a global major health threat without any significant change in 2016, and causes morbidity in 10.4 million with 90% adults and 65% male, and mortality among 1.7 millions of people including HIV patients (0.4 million deaths), and 56% were in five countries: India, Indonesia, China, the Philippines, and Pakistan. Moreover, there were 10% TB incident cases with HIV and 74% of these cases was found in Africa. Approximately 85 % of TB deaths among HIV positive and negative people occurred in Africa and South-East Asia regions. Furthermore, 95% of TB deaths were reported from resource-limited countries and it has been the leading cause of death from a single infectious agent, resulting to be above HIV/AIDS for the last 5 years (WHO, 2017).

While most morbidity and mortality from TB are preventable, the death toll from the disease is still unacceptably high and efforts to combat this must be continuously accelerated beyond the Sustainable Development Goal (SDG) and the End TB strategy targets, as the targets are reduction of TB deaths and incidence rate by 90% and 80% in 2030, compared with 2015 data. However, due to lack well-organizing health systems or TB control program such as getting a timely diagnosis and treatment, most of the TB cases are suffering (WHO, 2015B). As result, a

total of 87% of the incident TB were occurred in poor resource setting in South-East Asia (45%), Africa (25%) and Western Pacific (17%) WHO Region, but the remaining smaller (13%) proportions of cases occurred in Eastern Mediterranean (7%), European (3%) and Americas (3%) WHO Regions. The annual number of incident TB cases relative to population size varied widely among countries in 2016, from under 10 per 100 000 population in most high-income countries to 150–300 in most of the 30 high TB burden countries, and above 500 in a few countries including the Democratic People's Republic of Korea, Lesotho, Mozambique, the Philippines and South Africa (WHO, 2017).

Moreover, the 30 high TB burden countries accounted for 87% of all estimated incident cases worldwide, but 56% of the global incident was accounted for five countries from Asia continent such as India, Indonesia, China, the Philippines, and Pakistan (WHO, 2017). In 2016, WHO revised the definition of High Burden Countries (HBC) that three new HBC lists separately for TB, MDR-TB, MDR-TB is defined as resistance to both isoniazid and rifampin (Parsons *et al.,* 2011); and TB/HIV for the period 2016–2020. Each list contains 30 countries, these countries are defined as the top 20 in terms of the absolute number of estimated incident cases, plus the additional 10 countries with the most severe burden in terms of incidence rates per capita that do using the data of 2015. Each group accounts for 87–92% of the burden, with almost all of this accounted for by the top 20 countries in each group. A total of 48 countries appear in at least one list, and there are 14 countries in all three lists as summarized in figure 1.1 (WHO, 2017). Overall, the latest picture is one of a still high burden of disease, and of progress that is not fast enough to reach targets or to make major headway in closing persistent gaps.

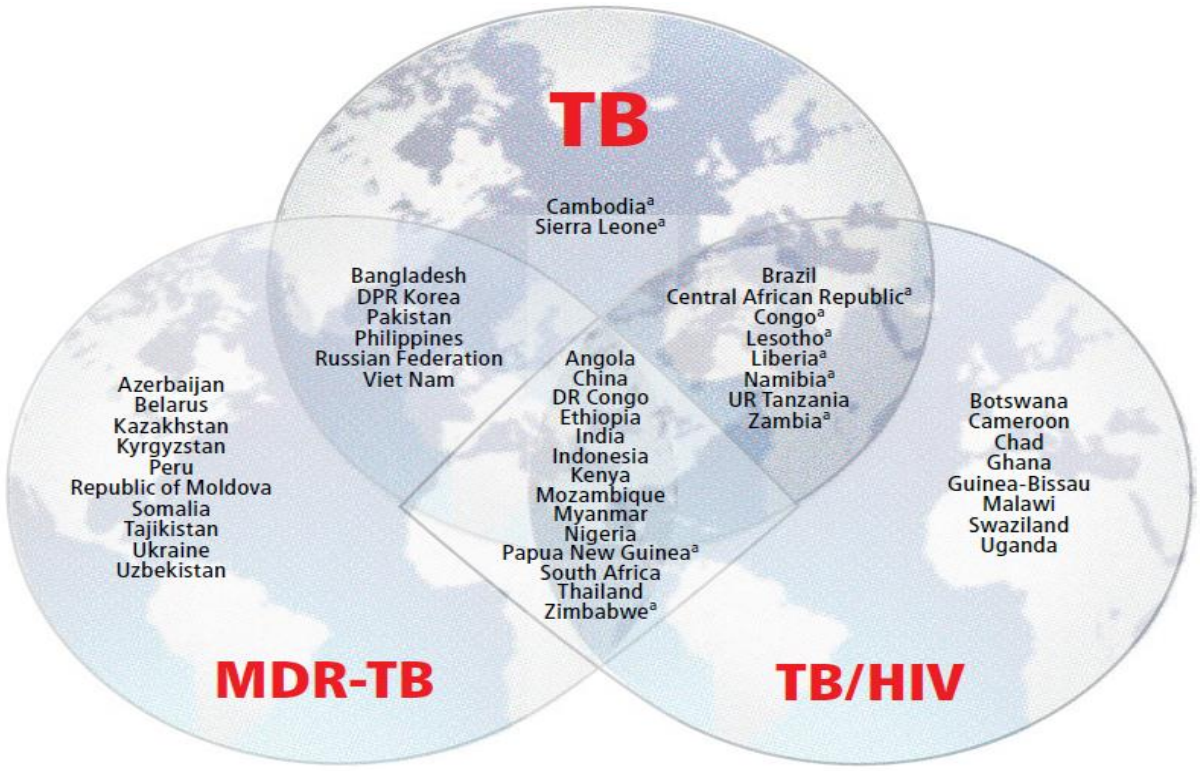


Figure 1-1: Countries in the three high-burden country lists for TB, TB/HIV and MDR-TB being used by WHO during the period 2016–2020, and their areas of overlap (Adopted from the World Health Organization (WHO, 2017).

[a] Indicates countries that are included in the list of 30 high TB burden countries on the basis of the severity of their TB burden (i.e. TB incidence per 100 000 population), as opposed to the top 20, which are included on the basis of their absolute number of incident cases per year.

1.4. Tuberculosis (TB) in Ethiopia

TB remains a major public health problem in sub-Sahara countries like Ethiopia. TB causes a disease among millions of people each year and it is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS (WHO, 2017). Ethiopia was among 27 high MDR-TB burden countries that carry 87% of the total global burden and one of the four countries in Africa (i.e. South Africa, Nigeria, and The Democrat Republic of Congo). The evidence, the WHO TB Global Report 2011 estimated the presence of 1600 (910-2800) MDR-TB among new TB cases and 580 (270-1000) among retreated TB cases, and a drug-resistant TB survey done in Ethiopia showed that there were 1.6% of new and 11.8 % of

retreatment MDR-TB cases (FDRE, 2012). However, the situation remains a major public health problem.

As a result, Ethiopia continues to be among the thirty high burden countries (TB, TB/HIV and MDR-TB), and TB in Ethiopia representing of one of the nation's leading causes of mortality in the country. According to WHO 2017 report, the prevalence and mortality rates in the country were 177/100,000 and 25/100,000 (among HIV negative TB cases) populations respectively, and the male to female ratio of cases for the same set of surveys shows a systematically higher burden of TB disease among men, with ratios ranging from 1.2 in Ethiopia for bacteriologically confirmed TB. Moreover, the prevalence of MDR-TB in Ethiopia was reported as 2.7% and 14% among new and previously treated cases respectively, moreover, the prevalence of TB/HIV co-infection was 8% (WHO, 2017). Moreover, several studies done in Ethiopia showed that the prevalence of MDR-TB was 31.4% in Jimma (Kedir *et al.,* 2015), 28% in Addis Ababa (Dawit *et al.,* 2008), 46.3% in Addis Ababa (Dereje *et al.,* 2012), 15.3% in Amara region (Daniel *et al.,* 2014), and 5% in Northwest Ethiopia (Tessema *et al.,* 2012). In addition, a report from WHO showed that the prevalence of multi-drug-resistant TB (MDR TB) was 2.3% and 17.8% among new TB and retreatment cases respectively (WHO, 2015). A rapid transmission MDR-TB is a major public health problem globally especially for resource-limited countries and become a major challenge for TB control program. In addition to this, a high prevalence of TB, poor treatment, limited access to health care, and several other related factors make MDR-TB difficult in the sub-Saharan region like Ethiopia (WHO, 2010a). Moreover, the emerging and rapid transmission of XDR-TB is also another challenge for TB control program (WHO, 2011a), XDR-TB is defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and at least one of the three second-line injectable drugs: kanamycin (KAN), amikacin (AMK), and capreomycin (CAP) (Parsons *et al.,* 2011).

Furthermore, according to Ethiopian Federal Ministry of Health report data also showed that TB is one of the leading causes of morbidity and the second most common cause of hospital death in Ethiopia. Besides (FMOH, 2012), the high prevalence of HIV and other infectious diseases, the low socioeconomic status of the people, overcrowding, and limited access to well-equipped health care facilities worsens the effect of MDR-TB. Besides, poor treatment outcomes, treatment costs, long time of treatment, and other related complications is creating MDR-TB a more complex disease than TB (WHO, 2010b). A study done in Ethiopia showed that long time of treatment, poor treatment follow up by a health worker, interruption of treatment and retreatment with the category II were a risk factor for developing drug resistance TB (Selamawit *et al.,* 2013).

1.5. Multi-drug resistance in *M. tuberculosis* and Associated Risk Factors

Over the past decades, *M. tuberculosis* became resistant to various anti-TB drugs, making infection control difficult. Drug-resistant TB threatens global TB care and prevention, and remains a major public health concern in many countries, especially in sub- Sahara countries. In Sub-Saharan Africa, the fight against TB has encountered a great challenge because of the emergence of drug-resistant TB strains and the high prevalence of other infection. Moreover, the onset of the HIV epidemic, and along with other factors such as immigration, and limited access to medical care led to increase TB incidence in many countries (WHO, 2012). Data from America and Europe showed that several outbreaks of MDR-TB among hospitalized HIV/AIDS patients were documented in the United States and in parts of Europe in the late 1980s and early 1990s (Moro *et al.,* 1998). As result, the outbreak of drug-resistant tuberculosis (DR-TB), particularly multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR - TB) is a challenge to TB control programs globally. Furthermore, treatment of MDR-TB is complicated; it requires the use of second-line anti-TB drugs which are less effective, more toxic and more costly. Moreover, treatment duration needs to be prolonged to 18-24 months (WHO, 2010b).

While resistance to drugs is a natural phenomenon, nevertheless, in *M. tuberculosis*, resistance to anti-TB drugs is considered man-made, as it commonly results from artificial selection of spontaneous drug resistance mutations during inadequate or incomplete therapy (Dheda *et al.,* 2010). Drug-resistant strains of MTB arise from spontaneous chromosomal mutations at a predictable low frequency but study revealed that drug-resistant TB development is mainly a result of unsuccessful TB control programs due to inadequate therapy, poor patient compliance, interrupted drug supply, and inappropriate treatment regimens, results in the emergence of resistant mutants (Gandhi *et al.,* 2006). Moreover, several similar studies conducted in different countries and settings revealed that inappropriate medical management, absence of directly observed treatment, lack of uniformity between public and private sectors, limited or interrupted drug supply, poor quality and widespread availability of anti-TBdrugs, were also reported as important causes associated with MDR-TB (Faustini *et al.,* 2006, WHO, 2010b). Over time, newer anti-TB drug resistance patterns have developed and increased the frequency of transmission of MDR and extensively drug-resistant (XDR) strains of *M. tuberculosis*, and it poses challenges for effective therapeutic options and infection control (WHO, 2011b).

In 2015, globally there were an estimated about 0.5 million new MDR-TB cases and an additional 0.1 million people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment, and 45% of the cases were found in India, China and the Russian Federation. Among newly this MDR-TB cases, only 20% were enrolled for treatment, and globally, the MDR-TB treatment success rate was 52% (WHO, 2016). However, the crisis of MDR-TB detection and treatment continues. In 2016, globally the estimated prevalence of MDR-TB was 4.1% among new patients and 19% among previously treated patients (see figure 1.2). There were an estimated 0.6 million incident cases of MDR/RR-TB in 2016, likewise, India, China and the Russian Federation continued as the countries with the largest numbers of MDR/RR-TB cases with 47% contribution to the global total cases. Moreover, there were about 0.24 million deaths

from MDR/RR-TB in 2016, similar to 2015 estimate. But only 22% MDR/RR-TB cases were started on treatment for drug-resistant TB, and globally, the MDR-TB treatment success rate was 54%, and remains low. The treatment success rate for HIV-associated TB was 78% and for extensively drug-resistant TB (XDRTB), it was 30% (WHO, 2017).

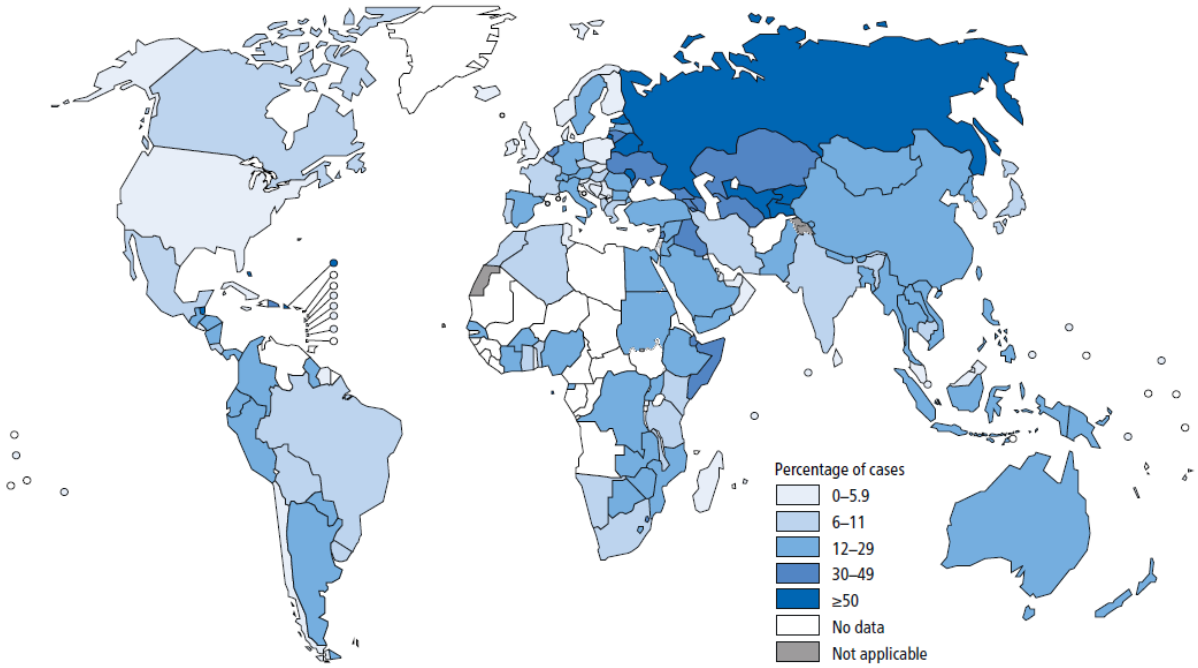


Figure 1-2: Percentage of previously treated TB cases with MDR/RR-TB in 2016, (Adopted from the World Health Organization) (WHO, 2017).

Furthermore, In Ethiopia, the low socioeconomic status of the people, a high prevalence of infectious diseases and limited access to well-equipped health care facilities worsens the effect of MDR-TB. Moreover, poor treatment outcomes, treatment costs, long time of treatment, and other related complications is creating MDR-TB a more complex disease than TB (WHO, 2010b). A study done by Selamawit, and collogues showed that long time of treatment, poor treatment follow up by a health worker, interruption of treatment and retreatment with the Category II were a risk factor for development drug-resistant TB (Selamawit *et al.,* 2013). According to a recent World Health Organization (WHO) report of high TB-related burden countries, Ethiopia was identified as being among the thirty highest TB-burdened nations (TB, TB/HIV and MDR-TB) with TB remaining one of Ethiopia's leading causes of mortality. According to 2017 WHO report, the

prevalence of MDR-TB in Ethiopia was reported to be 2.7% and 14% among new and previously treated cases respectively with the prevalence of TB/HIV co-infection assessed as being 8% of the affected population (WHO, 2017).

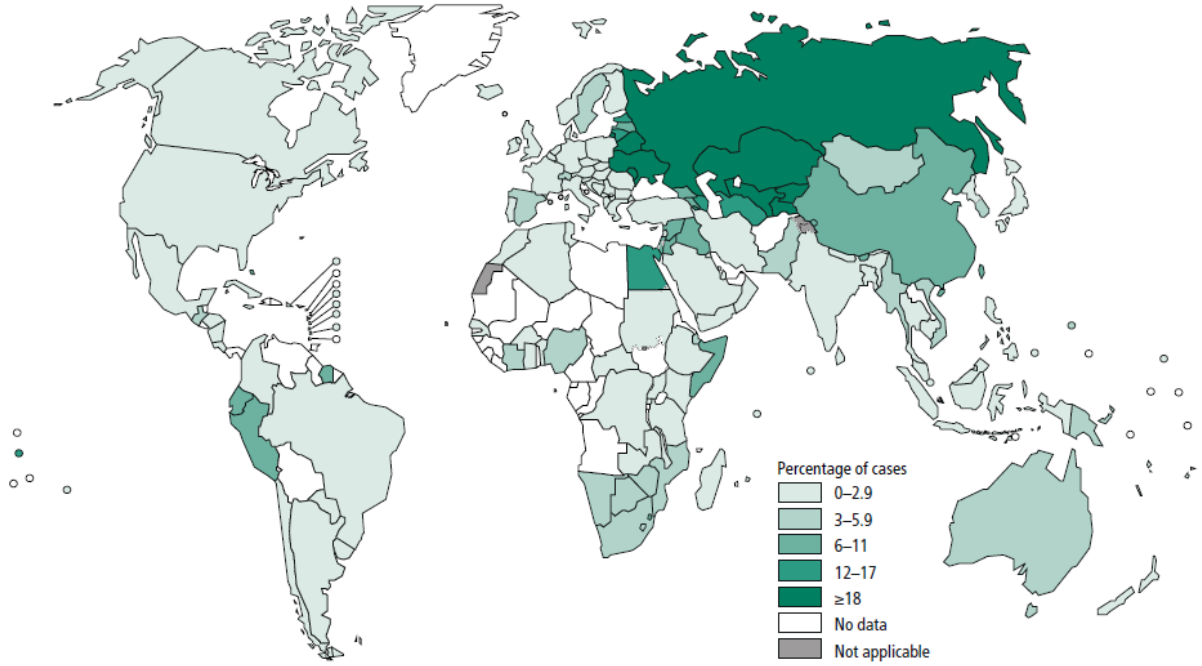


Figure 1-3: Percentage of new TB cases with MDR/RR-TB in 2016 (Adopted from the World Health Organization) (WHO, 2017).

1.6. Treatment of Tuberculosis

Anti-TBdrugs used for the treatment of TB are classified as first and second-line drugs. First line drugs include isoniazid (INH), rifampicin (RIF) ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STM). STM is no longer considered as a first line drug because of high rates of resistance. Second-line drugs include six classes of drugs: aminoglycosides e.g., amikacin (AMK) and kanamycin (KM); polypeptides e.g., capreomycin (CPM), viomycin and enviomycin; fluoroquinolones e.g., ciprofloxacin (CIP), levofloxacin (LFX), ofloxacin (OFX) and moxifloxacin (MXF); thioamides e.g. ethionamide (ETO) and prothionamide; cycloserine (CS); and paminosalicylic acid (PAS) (Lienhardt *et al.,* 2010). However, second-line drugs are less effective than the first-line drugs; have higher toxic side-effects; more costly, and unavailable in many developing countries. Furthermore, treatment of MDR-TB is complicated as it requires the use of

multiple a second-line anti-TB drugs simultaneously, with treatment duration having to be prolonged to 18-24 months (WHO, 2010b).

1.6.1 Treatment of drug susceptible TB

WHO has updated the treatment guidelines for drug-susceptible TB in April 2017. The updated guidelines include a recommendation to treat new pulmonary TB patients with drug-susceptible TB with a 6-month regimen as follow: 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by 4 months of isoniazid and rifampicin (2 INH, RIF, PZA EMB/4 INH, RIF). If the 8-month regimen using, it should be given for 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol, followed by 6 months of isoniazid and ethambutol (2 INH, RIF, PZA EMB/ 6 INH, EMB) for such patients is still in use, however WHO recommends that the 8-month regimen should be phased out. The WHO recommendations specify that daily therapy is preferred to thrice weekly dosing, and that fixed-dose combination tablets are preferred to single drug formulations (WHO, 2017).

1.6.2 Treatment of drug-resistant Tuberculosis

Previously, for MDR treatment, anti-TB drugs are grouped according to efficacy, the experience of use and drug class. The first-line anti-TB drugs (pyrazinamide, ethambutol, rifabutin) are in group 1, and the other injectable agents (Kanamycin, amikacin, and capreomycin) in Group 2. Fluoroquinolones were second-line drugs and classified under group 3, and the fourth group 4 was oral bacteriostatic second-line agents, such as para-aminosalicylic acid (PAS), cycloserine, terizidone, ethionamide, and protionamide. Moreover, group 5 were clofazimine, linezolid, amoxicillin/clavulanate, thioacetazone, imipenem/cilastatin, high-dose isoniazid, and clarithromycin drugs. The treatment regimens should consist of at least four drugs with either certain or almost certain, effectiveness (WHO, 2010b).

However, there are challenges for selection of drugs for the treatment of MDR-TB. Hence, WHO needs to update the guideline for the treatment of drug-resistant TB to respond to the challenge posed by multidrug-resistant and extensively drug-resistant tuberculosis. Based on the identified challenges the guideline was updated in 2016. The main focus of the update centered on the composition of treatment regimens for rifampicin-resistant (RR-TB) and multidrug-resistant TB (MDR-TB), and the shorter MDR-TB treatment duration, and the treatment of isoniazid-resistant and *M. bovis* TB. Drugs used in the design of longer MDR-TB treatment regimens are now regrouped differently based upon current evidence on their effectiveness and safety. MDR-TB treatment is recommended for all patients with RR-TB, regardless of confirmation of isoniazid resistance. According to the guideline, in patients with RR-TB or MDR-TB who were not previously treated with second-line drugs and in whom resistance to fluoroquinolones and second-line injectable agents was excluded or is considered highly unlikely, a shorter MDR-TB regimen of 9–12 months may be used instead of the longer regimens (WHO, 2016).

For treatment for patients with RR-TB or MDR-TB, treatment regimens should now consist of at least five effective anti-TB drugs during the intensive phase is recommended, including pyrazinamide and four core second-line TB drugs, as illustrated in table 1.1**,** one drug from Group A, one from Group B, and at least two drugs from Group C. If the minimum number of effective anti-TB drugs cannot be composed as given above, an agent from Group D2 and other agents from Group D3 may be added to bring the total to five. In patients with RR-TB or MDR-TB, it is recommended that the regimen be further strengthened with high-dose isoniazid and/or ethambutol. However WHO recommends that the shorter MDR-TB regimen not be used in patients who have been previously treated with second-line drugs for more than one month or who have documented or are likely to have strains resistant to drugs in the regimen. Preferably, resistance to at least fluoroquinolones and the injectable agent used in the regimen is excluded before starting treatment (WHO, 2016).

Table 1-1: Anti-TB drugs recommended for the treatment of RR-TB and MDR-TB

Group classification	Group Name		List of drugs by group
Group A.	Fluoroquinolones		Levofloxacin Moxifloxacin Gatifloxacin
Group B.	Second-line injectable agents		Amikacin Capreomycin Kanamycin Streptomycin
Group C.	Other core second-line agents		Ethionamide / prothionamide Cycloserine / terizidone Linezolid Clofazimine
Group D.	Add-on agents; they are not part of the core MDR-TB regimen	D1	Pyrazinamide Ethambutol High-dose isoniazid
		D2	Bedaquiline Delamanid
		D3	*p*-aminosalicylic acid Imipenem–cilastatind Meropenemd Amoxicillin-clavulanated (Thioacetazone)

1.7. Tuberculosis Diagnostics

Quality assured diagnostic facility is an important part of infection prevention and control program and it plays an important role in the control and prevention of TB through the timely detection and isolation of mycobacteria, species identification, detection of drug resistance, monitoring patient responses to therapy and epidemiological typing of *Mycobacterium* strains. The diagnosis of tuberculosis (TB) consists of both conventional methods (acid-fast microscopy, culture, biochemical identification, anti-TBdrug-susceptibility testing; DST) and modern molecular techniques. After the emergence of MDR-TB and XDR-TB, there is a great demand for accurate, reliable and rapid methods for diagnosing TB (WHO, 2011a).

In response to these facts, WHO has endorsed the use of 10 new diagnostics and approaches since 2007, including liquid culture, noncommercial culture methods, line-probe assays for DST, the light - emitting diode microscopy, and the Xpert MTB/RIF assay, and a decreasing in the

number of smears from three to two (WHO, 2011a). There are a number of scientific reports describing the development of methods employing various techniques for diagnosis *M. tuberculosis* TB and drug-resistant TB. Most of the current methods have been developed based on the principle of molecular techniques, and a wide range of diagnostic methods are available for detection of drug-resistant *M. tuberculosis*. As described below, the WHO recommends specific guidelines for the diagnosis of *M. tuberculosis.*

1.7.1 Conventional Methods

1.7.1.1 Microscopy

In many developing countries where TB prevalence is especially high, smear microscopy examination of sputum specimens still remains the primary, TB laboratory method available. Smear microscopy is a low-cost method which is effective in detecting the most infectious cases and can be performed in basic laboratories. Smear-positive TB patients have high bacterial loads and have already developed cavitary lesions in the lungs while patients with smear-negative pulmonary TB have lower bacillary loads. The limit of detection (LOD) for microscopy is 5,000-10,000 AFB per ml sputum. Compared to culture-positive sputum specimens, the sensitivity of smear microscopy is not more than it (Hepple *et al.,* 2012); however, the sensitivity of smear microscopy may be even lower in children and patients who are co-infected with HIV, as this cohort of patients typically has a lower bacterial load. Thus, many cases remain undiagnosed by standard laboratory method, since a sputum smear-positive for AFB may represent either *M. tuberculosis*, an NTM, or another AFB (Bishop and Neumann, 1970).

AFB resists acid-based decolorization procedures, hence the name acid-fast. The high mycolic acid content of the mycobacterial cell walls is responsible for the high retention of the dye. The most common staining method used to identify AFB is the Ziehl-Neelsen staining technique. The bacteria are first stained by carbolfuchsin combined with heat and then decolorized with acid-alcohol. Mycobacteria retain the color after decolorization with the acid, but other bacteria are

decolorized. The smear is subsequently counterstained with methylene-blue and analyzed in a light microscope. AFB are stained bright red and clearly stand out against a blue background (Madison *et al.,* 2001). AFB can also be visualized by fluorescence microscopy using specific fluorescent dyes (e.g. auramine-rhodamine stain), yielding better sensitivity (Van *et al.,* 2007). Although smear microscopy may be considered a reliable tool for detecting the most infectious TB patients and for monitoring patient response to therapy, the problem of drug resistance, cannot address with this technique.

1.7.1.2 Microbiological culture

Culture is considered the current diagnostic gold standard and is essential for detection of smear microscopy negative cases. Due to the particularly slow growth of *M. tuberculosis*, culture is a very time-consuming procedure. In fact, it may take weeks before colonies are obtained. Culture is compared to microscopy a relatively expensive method and requires specialized laboratories and highly trained personnel. Due to the pathogenic nature of *M. tuberculosis*, culture isolation is not suitable for laboratories that do not have a proper biosafety level, including necessarily e equipment. The advantage of culture tests over sputum microscopy is their higher sensitivity, allowing for the detection of very low numbers of bacilli approximately 10 bacilli/ml. The use of cultures increases the potential of diagnosing TB at early stages of the disease. Cultures are used for species identification and drug susceptibility testing [DST] (Miglior *et al.,* 2012).

Mycobacterial culture can be classified as a solid or a liquid medium. The yield of *M. tuberculosis* isolated from a liquid medium (e.g., Middlebrook 7H9) is greater than that from a solid egg-based medium (Lowenstein-Jensen [LJ]) or a solid agar-based medium such as Middlebrook 7H11 (Pfyffer and Palicova 2011). Globally, the egg-based culture medium Löwenstein-Jensen (LJ) is predominantly used for the isolation of *M. tuberculosis*. However, the agar-based culture medium Middlebrook is also used for culture. Both media require approximately three to six weeks from inoculation to show visible colonies (Naveen and Peerapur, 2012). Mycobacteria have a slightly

faster growth rate in liquid media compared to solid media, consequently culturing methods involving liquid growth medium allows a more rapid identification of the bacteria (Chihota *et al.,* 2010). Automated liquid culture systems, such as the Bactec MGIT system (BD) or the BacT/Alert (bioMérieux), provide continuous monitoring for mycobacterial growth and, studies, significantly improve the recovery of *M. tuberculosis* as well as reduce the time to detection compared to a solid medium culture (Brittle *et al.,* 2009).

1.7.1.3 Species identification

Differentiation of *M. tuberculosis* from other mycobacteria represents an important health issue in the diagnosis of TB. NTMs are increasingly recognized as causative agents of opportunistic infections in humans. The classical laboratory approach to the determination of bacterial species involves phenotypic characterization. Species identification is performed by observing colonial morphologic features, pigment production, growth rate and by employing biochemical tests for studying enzymatic characteristics. Biochemical tests involve for example investigation of reduction of nitrate and potassium tellurite, catalase production, and hydrolysis of Tween. Biochemical tests have successfully been used since the 1950s (Hall *et al.,* 2006).

1.7.1.4 Drug Susceptibility Testing

There are two different methods to perform drug susceptibility for *M. tuberculosis*, they are phenotypic methods and genotypic methods. Phenotypic methods assess the inhibition of *M. tuberculosis* growth in the presence of antibiotics and define resistance based on the response of the organism when exposed to the drug. Genotypic methods are based on the detection of genes or mutations known to be associated with resistance. Conventional drug susceptibility testing (DST) utilizes phenotypic methods and depends on a variety of factors, the first of which is the definitive microbiologic diagnosis of *M. tuberculosis* with the isolation of the organism (Woods, 2011). Though the phenotypic method is slow, it provides the complete susceptibility profile of *M. tuberculosis*.

Phenotypic DST methods are performed as direct or indirect tests in solid or liquid media. Direct testing involves inoculating drug-containing and drug-free media directly with a concentrated specimen. Indirect testing involves inoculating drug-containing media with a pure culture grown from the original specimen. The indirect proportion method is considered as the gold standard for defining resistance. It requires weeks to complete due to the slow growing nature of *M. tuberculosis*. The growth of the organism in the control medium is compared to growth on drug-containing media to determine susceptibility or resistance. If more than 1% of the organisms in a population are resistant to a drug, the strain is considered resistant. It is due to the survival of a small number of bacteria during antibiotic exposure despite lacking genetic resistance mechanisms that the minimum inhibitory concentration (MIC) is commonly defined as the lowest concentration of antibiotic that kills or inhibits the growth of 99% of a bacterial population. As is the case with *M. tuberculosis* organism detection, automated liquid culture systems reduce the turnaround time for detection of *M. tuberculosis* resistance. However, the time to result is still quite slow, taking 10 to 14 days (WHO, 2012).

Moreover, molecular methods for the detection of drug resistance can be divided into 2 major categories, probe-based methods and sequence-based methods. The major advantage of the sequence-based methods is their ability to provide sequence information and reveal the identity of the mutation. Because mutations are not always associated with resistance, availability of mutation identity enables proper interpretation of Molecular drug susceptibility testing (MDST) results. When a new mutation is detected whose association with resistance is unknown, the interpretation should be deferred to culture-based drug susceptibility testing (CDST) results, and the risk of reporting false resistance can be avoided. In addition, the level of drug resistance may vary with different mutations. The availability of mutation identity offers an opportunity to study drug MIC for each specific mutation. The accumulation of this knowledge enables prediction of

drug resistance levels for each mutation. However, this will not be possible if the mutation identity is not provided (Shou-Yean *et al.,* 2014).

1.7.2 Molecular Methods for Diagnosis of TB and Drug-Resistant TB

Molecular biology is now becoming more important in the diagnosis of mycobacteria. It supports culture either by serving as a rapid direct test on specimens or by enabling a rapid and unequivocal species differentiation from culture material. Nucleic-acid-based methods have largely displaced the classical methods. Molecular genetic tests offer considerable time advantages in the identification of mycobacteria, enabling a more rapid initiation of resistance tests and specific treatment. They are useful tools for the detection and differentiation of mycobacteria from cultures and can have a high specificity and sensitivity. There are several methods for diagnosis *M. tuberculosis* TB and drug-resistant TB. Most of the methods have been developed based on the principle of molecular techniques; the molecular method principles are based on PCR amplification of a specific chromosomal region followed by analysis of the PCR product for detection of mutations associated with resistance to a particular drug. The presence or absence of a specific mutation is then regarded as an indication that the investigated isolate is susceptible or resistant to a particular drug (Henegariu *et al.,* 1997). Even though there are several diagnostic methods available for diagnosis of TB, WHO recommended methods are described below.

1.7.2.1 Line Probe Assays

Molecular line probe assays (LPAs) apply principles of nucleic acid amplification to detect both species and mutations associated with drug resistance. In these assays, similar to sequencing technologies, a genomic region is first subjected to PCR with biotinylated primers, followed by hybridization of the PCR product to oligonucleotide probes immobilized on a nitrocellulose strip. The presence of a mutation prevents hybridization of the PCR product to wild-type probes, and conversely, a wild-type sequence prevents hybridization to the mutant-specific probes. The

biotinylated product enables visualization of colored bands by the naked eye. Mutations are detected by lack of hybridization to wild-type probes, as well as by hybridization to specific probes designed for the most commonly occurring mutations. However, in some cases, a complete loss of signal is not observed when a mutation is present, but rather just a decrease of band intensity. The WHO endorsed LPAs for the detection of mutations associated with drug resistance in *M. tuberculosis* in 2008 (WHO, 2008b).

The GenoType MTBDRplus assay detects, apart from RIF resistance, mutations in katG and the promoter region of inhA, allowing simultaneous detection of INH resistance. Thus, the GenoType MTBDRplus assay enables detection of MDR-TB. The GenoType MTBDRsl assay is developed for detection of fluoroquinolones (e.g. ofloxacin and moxifloxacin) resistance by targeting the gene gyrA, and aminoglycosides/cyclic peptides (injectable antibiotics as capreomycin, viomycin/kanamycin, amikacin) by analysis the gene rrs, enabling detection of Extensively Drug-resistant Tuberculosis (XDR-TB). Analysis of the gene embB, linked to resistance to the first-line drug EMB, is also included in the GenoType MTBDRsl assay. The GenoType MTBDRplus assay is also optimized for smear-negative pulmonary clinical specimens. All procedures are identical and are divided into three steps: DNA extraction, a multiplex amplification using biotinylated primers, and reverse hybridization (Hillemann *et al.,* 2009). In general the evaluation of the performance LPA results that compared conventional DST methods showed that the sensitivity of LPA is more than 97% with 99% specificity for detecting rifampicin resistance, moreover the sensitivity of LPA with combination of rifampicin and isoniazid is more than 90% with 99% specificity in *M. tuberculosis* isolates (WHO, 2008).

1.7.2.2 GeneXpert MTB/RIF

In December 2010, WHO recommended the use of the Xpert MTB/RIF assay for diagnosis of M. tuberculosis and RIF drug resistant TB. GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA;) is a rapid, automated, and cartridge-based Nucleic acid amplification (NAA) test based on

real-time PCR test that can detect TB along with rifampicin resistance directly from sputum within 2 hours of collection. The GeneXpert cartridges are pre-loaded with all of the necessary reagents for sample processing, DNA extraction, amplification, and laser detection of the amplified rpoB gene target. The assay is designed to detect the MTC as well as RIF resistance (WHO, 2013b). Species identification and mutation detection are achieved through analysis of hybridization of five molecular beacons to the PCR product, specifically targeting the RRDR of *rpoB.* The primers amplify a portion of the rpoB gene containing the 81 base pair "core" region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that is associated with RIF resistance.

The sensitivity of the GeneXpert MTB/RIF assay for detecting TB is similar to that of liquid culture with sensitivity (88%). When compared with liquid culture as a reference standard the specificity is also high (99%). For smear-negative culture-positive TB, the pooled sensitivity of Xpert MTB/RIF has been found to be 68%. The superior performance of Xpert MTB/RIF in detecting TB over that of microscopy makes it a particularly useful tool for case-finding among people living with HIV. As a tool for detecting rifampicin resistance, Xpert MTB/ RIF has a sensitivity of 95% and specificity of 98% when compared with phenotypic reference standards. While it should be noted that mono-resistance to rifampicin is found in approximately 5% of rifampicin-resistant strains, a high proportion of rifampicin resistance is associated with concurrent resistance to isoniazid (95%). Thus, detecting resistance to rifampicin can be used as a marker for MDR-TB with a high-level of accuracy (WHO, 2013b).

1.7.2.3 Nucleic acid hybridization test (NAAT)

Currently, the AccuProbe Mycobacterium tuberculosis Complex Culture Identification Test Kit, (Gen-Probe Incorporated, San Diego, CA, USA), a Nucleic Acid Hybridization Test is widely used for identification of MTB complex. Although the test has a limitation in that does not differentiate between members of the MBTC, the test result of MTBC is sufficient for treatment purpose. The

principle of nucleic acid hybridization test, use of a single-stranded DNA probe with a chemiluminescent label is complementary to the ribosomal RNA of the target. After the ribosomal RNA of the bacteria is released, the labeled DNA probe combines with the target RNA to form a stable DNA:RNA hybrid, which can be measured in an illuminometer. The decision to test a culture with an MTB complex probe may also depend on the appearance under the microscope (in a liquid medium) and the pigmentation of the bacteria or the pellet. (ECDC, 2016). Compared with culture, NAAT can detect the MTB in a specimen weeks before culture for 80%–90% of patients suspected to have pulmonary TB whose TB is ultimately confirmed by culture. Although NAAT testing is recommended to perform the initial diagnosis of persons suspected to have TB, the currently available NAAT tests should not be ordered routinely when the clinical suspicion of TB is low given that the positive predictive value of the NAAT test is <50% for such cases (CDC, 2009).

1.7.3 Chromatographic immunoassay

Chromatographic immunoassay is a qualitative method for detection of *M. tuberculosis* complex from cultures. A definite diagnosis of TB can be made by identifying *M. tuberculosis* complex organisms from a clinical sample after growth in solid or liquid media. Since *M. tuberculosis* complex strains with the exception of some sub-strains of M. bovis BCG but not non-tuberculous mycobacteria specifically and predominantly secrete the MPB64 protein (mycobacterial protein fraction from BCG of Rm 0.64), this can be used to discriminate between *M. tuberculosis* complex and non-tuberculous mycobacteria. The testing method is based on immune-chromatographic principles, in which antibodies labeled with colloidal particles (such as colloidal gold) react with target antigens to form a migrating antigen-antibody complex, which is captured by a second fixed antibody. A color reaction takes place where the labeled particles are fixed (ECDC, 2016).

1.7.4 Challenges of TB Diagnosis

Although there are several advanced and classical methods for diagnosis of TB in suspected patients, TB diagnostics are still suboptimal in performance, especially for smear-negative TB due to lack of appropriates specimen for diagnosis and present with many different symptoms. Up to 50% of patients with PTB are smear-negative (Behr *et al.,* 1999), and in patients suspected of having TB with smear-negative, culturing remains essential for diagnostic testing. Moreover, HIV is one of the most important risk factors associated with an increased risk of TB infection progressing to active TB disease and HIV-infected patients have an annual risk of up to 15% of developing active TB once infected. Research has indicated that more than 60% of new cases of PTB in developing countries are co-infected with HIV (Corbett *et al.,* 2003*)*. Although there is a high prevalence of TB among HIV patients, they are not able to produce sputum for diagnostic testing (Peter *et al.,* 2012). Accordingly, diagnosis is challenging and often delayed, as indicated in research that reported that autopsy examinations revealed a large burden of undiagnosed TB in HIV-infected hospitalized patients (Cox *et al.,* 2010).

In sputum-scarce PTB cases, bronchoalveolar lavage fluid and gastric lavage are a preferred clinical specimen in adults and children respectively, but, bronchoalveolar and gastric lavage can be not obtained with a simple procedure and only found in a well-equipped healthcare setting and have very low detection rates (Behr *et al.,* 1999). However, there are studies that have reported positively on the utility of GeneXpert MTB/RIF for diagnosis PTB using urine (Theron *et al.,* 2011, Peter *et al.,* 2012). Moreover, other studies have revealed that the detection rate of PTB was improved by using urine specimen from sputum-scarce TB cases (Torrea *et al.,* 2005, Gopinath and Singh, 2009). As Colby postulated mycobacterium tubercle could be excreted through the kidneys and the microorganism could be found in the urine of TB patients who have no symptoms pertaining to the urinary tract (Colby 1961). Additional studies done by Kafwabulula *et al.*(2002) and Aceti *et al.,* (1999) supported this assumption. Furthermore, another study showed that the

detection rate of PTB was increased among culture-negative PTB cases by using urine (Torrea *et al.,* 2005). As a result, acquiring a diagnostic specimen remains a major hindrance in sputum scarce patients suspected of having active PTB. Therefore, novel diagnostic approaches must be considered as means to provide a rapid diagnosis of active PTB in patients for whom sputum samples are not available.

1.8. Methods for Genotyping of *M. tuberculosis*

Genotyping of *M. tuberculosis* has been used in molecular epidemiology since 1990. Molecular epidemiological investigations have contributed significantly to the understanding of the epidemiology and control of tuberculosis by providing information on transmission dynamics (Tostmann *et al.,* 2008), determining the importance of reactivation versus exogenous reinfection, investigating/confirming outbreaks (Paranjothy *et al.,* 2008), estimate the proportion of epidemiologic links not identified by conventional contact tracing (Tostmann *et al.,* 2008), confirming laboratory cross-contamination and to identify the clonal spread of successful clones, including multi-drug-resistant ones (Mathema *et al.,* 2006). In addition, an important study concluded that molecular markers can be used to evaluate host- and strain-specific risk factors and possible genotype specific differences in phenotypes such as virulence and transmissibility (Garcia *et al.,* 2008).

The gold standard method for genetic analysis of *M. tuberculosis* is insertion sequence IS6110-based restriction fragment length polymorphism (RFLP) analysis (Choi *et al.,* 2010). However, there are similar discriminatory power and greater convenience that can be demonstrated with other methods such as spacer oligonucleotide typing (spoligotyping), which detects 43 spacer sequences interspersed with direct repeats (DRs) in the genomic region of *M. tuberculosis* complex (Kamerbeek *et al.,* 1997); mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) that uses PCR amplification and gel electrophoresis to determine the number and size of tandem repetitive DNA sequence in the *M. tuberculosis* genome. A MIRU-

VNTR analysis use variations in the copy of repeats in highly variable regions of the MTC genome to show changes in the genome over relatively shorter time periods (Supply *et al.,* 2006). Whole Genome Sequencing (WGS), a recent method with high discriminatory. A recently study indicates that MIRU-VNTR typing has an equal discriminatory power with IS*6110*-RFL, and that the combination of MIRU-VNTR typing and spoligotyping is an appropriate means of obtaining reliable and accurate genotyping results (Jonsson *et al.,* 2014). Thus, 3 important and widely used DNA fingerprinting methods such as spoligotyping, MIRU-VNTR, IS6110 RFLP typing, were described below.

1.8.1 Spoligotyping

Spacer oligonucleotide typing (spoligotyping) is the most commonly used PCR-based technique for studying the phylogeography of MTBC. Spoligotyping is based on polymorphisms in the direct repeat (DR) locus of the mycobacterial chromosome. This method utilizes the amplification of variable DNA spacers in the genomic DR region of MTBC isolates as this target shows considerable strain-to-strain polymorphisms (De Jong *et al.,* 2009). The well-conserved 36-bp direct repeats are interspersed with unique spacer sequences, varying from 35 to 41 bp in size. The order of the spacers has been found to be well conserved. Currently, 94 different spacer sequences have been identified, 43 of which are used in the first-generation spoligotyping for MTBC strains conserved (Van Embden *et al.,* 2000). After amplification, the denatured PCR products are applied in the reversed line on a membrane with covalently bound multiple synthetic spacer oligonucleotides deduced from DR region sequences of the two control strains (*M. tuberculosis* H37Rv and *M. bovis* BCG P3).

An important advantage of spoligotyping is its genuine sensitivity estimated at 10 fg of chromosomal DNA, equivalent to DNA from 2-3 bacterial cells, Spoligotyping can use directly clinical specimens without culture besides, spoligotyping can also use nonviable cultures. The results are highly reproducible, and the binary (present/ absent) data generated can be easily

interpreted and computerized and are amenable to interlaboratory comparisons (Jagielski *et al.,* 2014). However, Spoligotyping has a lower level of discrimination than the IS*6110* RFLP typing and using of Spoligotyping alone is not sufficient for epidemiological linking studies (Kremer *et al.,* 2005). The reason for the limited discriminatory capacity of the spoligotyping method is due to the fact that it targets only a single genetic locus, covering less than 0.1% of the *M. tuberculosis* complex genome. Moreover, spoligotyping cannot detect contaminated isolates and multistrain infections directly from clinical specimens and required expensive equipment.

1.8.2 Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeat (MIRU-VNTR) Typing

M. tuberculosis was among the first bacterial species in which tandem repeat loci were identified, and it was called a variable number of tandem repeat (VNTR) loci. VNTR sequences have emerged as valuable markers for genotyping of various bacterial species. VNTR typing makes use of the variability in the numbers of repeats present at particular known tandem repeat loci in bacterial genomes. The methodology comprises PCR amplification using primers specific for the regions flanking tandem repeat loci and the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, the sizes of the PCR products reflect the number of repeats in each VNTR locus. The final result is a numerical code, corresponding to the number of tandem repeats present in each VNTR locus and this serves as a DNA fingerprint of a bacterial isolate. Supply *et al.,* identified 41 VNTR of mycobacterial interspersed repetitive units (MIRU) as 46 to 101 bp tandem repeats scattered at 41 loci throughout the chromosome of MTB H_{37}Rv (Supply *et al.,* 1997).

Although VNTR typing was introduced with 12 or 15 MIRU-VNTR loci, it has a limited level of discrimination (Smittipat *et al.,* 2005). However, an improved MIRU-VNTR typing based on 24 MIRU-VNTR loci method was introduced in 2006. That made it possible to distinguish unrelated strains and provided the clonal stability to reliably identify isolates from the same transmission

chain. MIRU-VNTR typing of bacterial strains has proven to be a suitable method for the detection of genetic polymorphisms within bacterial species (Supply *et al.,* 2006). In addition using of additional 3 hypervariable loci (VNTRs 3232, 3820, and 4120) is recommended as a second-line typing step, particularly to differentiate Beijing genotype strains (Jagielski *et al.,* 2014). The MIRU-VNTR typing based on 24 MIRU-VNTR loci method accounts for 96% of all detected polymorphisms in MTB strains. The turn-around time for this typing method is much shorter and easier, and easy-to-compare results format when compared to RFLP typing.

1.8.3 Restriction Fragment Length Polymorphism (RFLP) Typing

The RFLP method for typing bacterial strains has been the most extensively used method over a decade because of its high-level of discrimination and reproducibility, and the fact that this was the first method that proved to be a suitable DNA-strain typing method for studying transmission. Moreover, IS*6110*-RFLP typing has a characteristic of allowing distinguishing epidemiologically related from unrelated isolates. It is based on the fact that the number of IS6110 insertion sequences present in the genome of strains differs from 0 to approximately 30. These genomic insertion sites themselves are also highly variable in MTBC strains, resulting in highly variable banding patterns (Van Soolingen *et al.,* 2001). The insertion sequence of IS6110 is present at multiple copies in most members of the complex except in *M. bovis which* normally contains only one copy of sequence. The copy number of IS*6110* ranges from 0 to 25 and depends on the frequency of transposition, which is largely conditioned by the nature of the genomic region at which transposition occurs. IS*6110* can be integrated into any place of the chromosome but there are regions with a higher frequency of transposition.

They are usually located within coding regions of MTB DNA. Variation in the copy number and locations within the genome are responsible for the high degree of IS*6110* polymorphism and have predisposed this sequence to be used as a specific molecular marker for genotyping of *M. tuberculosis* strains (Wall *et al.,* 1999). The RFLP is technically demanding and time consuming

and it needs 2 micrograms of highly purified genomic DNA. The process involves restriction enzyme cleavage of the DNA with *Pvu*II restriction enzyme that cleaves the IS*6110* sequence only once; fragment separation by electrophoresis; then transfer the fragments to a DNA membrane and hybridization by a labeled probe, and final visualization of the results on a light-sensitive film. As a result, every visualized fragment represents a single copy of IS*6110* surrounded by different in length flanking DNA. Each individual step of the process is crucial for the final result, which also gives an idea of the difficulties experienced with regard to inter-laboratory comparability (Van Embden *et al.,* 2000).

1.9. Molecular Epidemiology of *M. tuberculosis*

The MTBC is genetically monomorphic with a high-level of genomic sequence similarity, limited horizontal gene transfer, and a clonal population structure (Achtman 2008). This apparent homogeneity led to the assumption that genetic diversity among MTBC strains would not be of clinical significance. However, data from molecular genotyping showed that there was a diverse population structure of *M. tuberculosis* with several geographically-associated lineages (Hershberg *et al.,* 2008). The lineage distribution among cases caused by *M. tuberculosis* exerts distinct geographical association worldwide (Hershberg *et al.,* 2008, Coscolla and Gagneux, 2014). While lineages 1 (The Philippines and Rim of Indian Ocean) and 3 (India and East Africa) are prevalent in East Africa, Central, South- and South-East Asia, and lineages 2 (East Asia) and 4 (Europe, America, and Africa) are the most widely distributed worldwide. Lineages 5 and 6, which are also known as *M. africanum* West Africa 1 and West Africa 2, respectively, are localized in West Africa (De Jong *et al.,* 2010). Lineage 7 is a *M. tuberculosis* lineage recently discovered in north-western Ethiopia (Firdessa *et al.,* 2013, Comas *et al.,* 2015).

Moreover, MTBC and the human host have had a co-evolutionary relationship spanning several centuries. It is presumed that *M. tuberculosis* originated in Africa and co-evolved into modern lineages with the out-migration of humans from Africa (Galagan, 2014). The recent global

distribution of *M. tuberculosis* lineages showed that the Beijing strain was the most prevalent in East Asia region and found more than 50% of all strains. Likewise, it was the most prevalent genotype in Asia however, the Beijing strain was less prevalent in Africa, Australasia, North America, Europe and South America regions (Jagielski *et al.,* 2016). Moreover, the prevalence of geographically-associated lineages were found in African (Uganda, Cameroon and S-type), Asian (Beijing and CAS), Latin American-Mediterranean (LAM), African- European populations (X-type, Ghana and Haarlem) and East African- Indian (EAI) lineages that can also be further subdivided into well-defined genotypes (Hershberg *et al.,* 2008).

In Ethiopia, there are only a few studies done using combined spoligotyping and MIRU-VNTR typing method (Tessema *et al.,* 2013, Fantahun *et al.,* 2015, Solomon *et al.,* 2015), and these studies were done in the region of Amhara, Ethiopia. However, several studies were done using spoligotyping method (Mulalem *et al.,* 2010, Amare *et al.,* 2012, Adane *et al.,* 2012. Garedew *et al.,* 2013, Mulugeta *et al.,* 2014). A study done by Tessema *et al.,* (using combined spoligotyping and MIRU-VNTR typing method) in Ethiopia showed that the 38.9% of Isolate was Dehli/CAS, with 8.6% Haarlem. Ural, and LAM were found to be 3.3% of each, and 31.6% of the isolates appeared to identify four previously undefined lineages. Of these 13.1% isolates were labeled Ethiopia_3, followed by 7.8% Ethiopia_1, and 7.0% Ethiopia_H37Rv like (Tessema *et al.,* 2013). In addition a study done by Fantahun *et al.,* (2015) showed that majority of the strains were Delhi/CAS (38.8%) followed by Ethiopia_1 (9.7%), Ethiopia 3_ (8.7%), and Ethiopia H37RV-like (8.2%), Ethiopia_2 (7.7%), and Haarlem (7.7%). A small percentage of URAL, Uganda l, LAM, S-type, X-type, TUR, EAI, and Beijing genotype were identified.

Another similar study done by Firdessa *et al.* (2013) in Ethiopia showed that 71% of the isolates belonged to lineage 4, which was the most common lineage in Ethiopia. Lineage 3 was found to be prevalent with 18.2% of the isolates, with Lineage 3 being the most prevalent (47%) in the northern part of Ethiopia. Moreover, 1.6% of the strains belonging to lineage 1 were found in the

southern region. Further, 5.4% isolates were identified as members of a lineage 7. Moreover another previous study (done by spoligotyping method) from Ethiopia showed that the there were three dominant linages which were T (41%), Manu (19%), and CAS (16%), with four additional lineages being less prevalent X (5.7%), Haarlem (3.9%), EAI (3.8%), and LAM (2.9%) (Mulugeta *et al.,* 2014). Another similar study among MDR-TB patients showed that about 96% of the isolates were found to be T and CAS families (Mulalem *et al.,* 2010). Furthermore, a study done by Amare and colleagues in Southwest Ethiopia showed that 64.7% isolates belonged to T, Haarlem and Central-Asian (Amare *et al.,* 2012).

1.10. Anti-TB Drugs and Mechanisms of Drug Resistance

After the introduction of the first anti-TB drugs, resistance to streptomycin (STR), para-aminosalicylic acid (PAS), and isoniazid (INH) was observed in clinical isolates of *M. tuberculosis* (Mitchison 1985). According to a recently published review on the mechanisms of drug resistance in *M. tuberculosis* by Zhang and Yew (2015), there are two types of mechanism of drug resistance in *M. tuberculosis*: genetic resistance and phenotypic resistance. Phenotypic resistance or drug tolerance is due to epigenetic changes in gene expression and protein modification that cause tolerance to drugs in non-growing persister bacteria, while genetic drug resistance is due to mutations in chromosomal genes in growing bacteria. The genetic resistance to the anti-TBdrug due to spontaneous chromosomal mutations is found to be at a frequency of 10^{-6} to 10^{-8} mycobacterial replications.

A point mutation is an alteration in the DNA sequence caused by a single nucleotide base change, deletion, or insertion. In the case when a gene code for an amino acid occurred due to substitutions, which can be classified as missense or silent mutations. Missense mutations (nonsynonymous) are those that alter the amino acid sequence, and in the reversed scenario; silent mutations (synonymous) are those that do not alter the amino acid sequence. Although base substitutions are the most commonly confer drug resistance in *M. tuberculosis*; insertions

and deletions of single bases, or longer regions, are also identified in among drug resistance *M. tuberculosis* (Sandgren *et al.,* 2009). Several microorganisms use different mechanisms to resist the action of antimicrobial drugs and *M. tuberculosis*, like other bacteria, becomes resistant by a number of strategies. The best understood of these mechanisms are hydrophobic cell wall, drug efflux systems, drug inactivating enzymes and inactivation of drug-activating enzymes. The mechanisms for development of drug resistance are broadly classified as intrinsic and acquired drug resistances (da Silva *et al.,* 2011) described below.

Intrinsic drug resistance

Intrinsic drug resistance is the innate ability of a bacterium to resist the activity of a particular antimicrobial agent through its inherent structural or functional characteristics. An intrinsic drug resistance in *M. tuberculosis* has been attributed to its unique cell wall properties, including the presence of mycolic acids, which constitute a very hydrophobic barrier responsible for resistance to certain antibiotics (Karakousis *et al.,* 2004). In addition, MTB possesses β-lactamase enzymes, which confer intrinsic resistance to β-lactam antibiotics and recently, the role of efflux mechanisms has also been recognized as an important factor in the natural resistance of mycobacteria against antibiotics such as fluoroquinolones and aminoglycosides.

Acquired drug resistance

Acquired drug resistance is the ability of microbes to resist the activity of a particular antimicrobial drug to which it was previously susceptible. Unlike the situation in other bacteria where acquired drug resistance is generally mediated through horizontal transfer by mobile genetic elements, such as plasmids and transposons, in *M. tuberculosis,* acquired drug resistance in *M. tuberculosis* complex is caused mainly by spontaneous mutations in chromosomal genes, and the selective growth of such drug-resistant mutants being promoted during suboptimal drug therapy (Kolyva and Karakousis 2012). The development of drug resistance in TB summarized in figure 1.4 and is classified as acquired resistance when drug-resistant mutants are selected as a result of

ineffective treatment or as primary resistance when a patient is infected with a resistant strain. Studies showed that the rate of a mutation depends on the nature of the drug selection, but for most of the main anti-TB drugs, this occurs at a rate of at a frequency of 10^{-6} to 10^{-8} mutations per mycobacterial replications (Zhang and Yew, 2009).

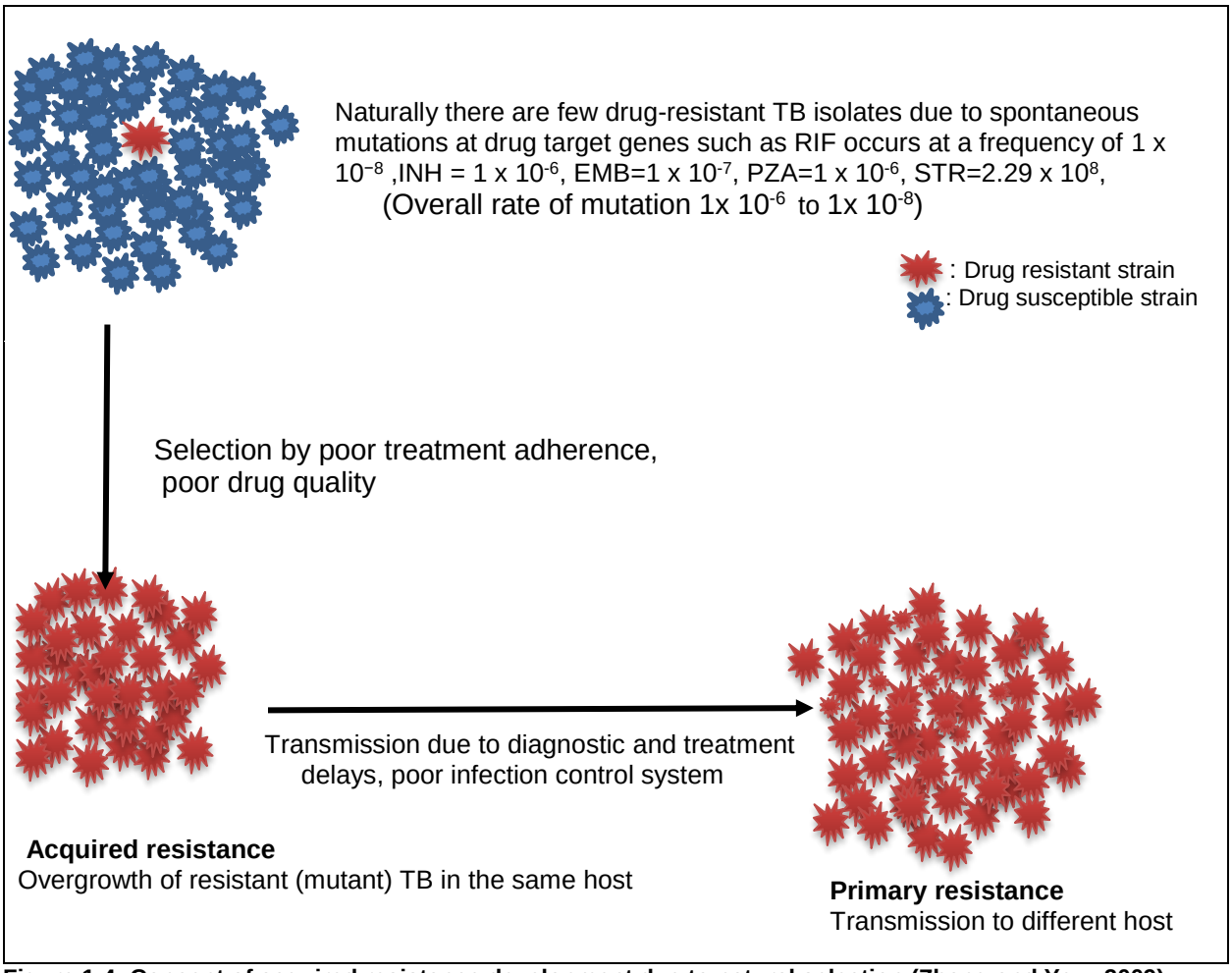


Figure 1-4: Concept of acquired resistance development due to natural selection (Zhang and Yew, 2009).

However, the current growth of a drug-resistant TB patient population has resulted in significant levels of concern associated with the prospects for success of global control of TB. (Dye and Williams, 2010). Reports indicate that up to 22.3% of MDR cases demonstrating resistant to various classes of anti-TB drugs (Sharma and Mohan 2004) that, on a global basis, we can expect as many as 40,000 new cases of XDR-TB each year (Dye and Williams, 2010). Even if most drug-resistant TB arises from spontaneous chromosomal mutations, studies indicated that selection

pressure, selection pressure is any phenomena which alters the behavior and fitness of living organisms within a given environment, that is caused by misuse of anti-TB drugs, such as inadequate therapy, poor patient adherence to treatment, and inappropriate treatment regimens, will increasingly represent a significant contribution to the emergence of resistant mutants. Moreover failure to detect drug-resistant TB results in increased mortality and further transmission of drug-resistant TB in the population (Gandhi *et al.,* 2006). The molecular mechanisms of resistance and drug action of the current available anti-TB drugs are discussed below.

1.10.1 Molecular Mechanisms of Resistance and drug action to First-Line TB Drugs

Any drug used in the anti-TB regiment is supposed to have an effective sterilizing activity that is capable of shortening the duration of treatment. Currently, a four-drug regimen is used consisting of INH, RIF, pyrazinamide (PZA) and ethambutol (EMB) and in cases calling for retreatment case streptomycin (STR) is prescribed (WHO, 2010b). Resistance to first-line anti-TB drugs has been linked to mutations in several genes such as *katG, inhA, ahpC, fabG* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance (Figure 1.5).

1.10.1.1 Rifampicin (RIF)

Rifampin, rifapentine, and rifabutin have been commonly used as first-line therapies in combination with other drugs for the treatment of TB infections. It was introduced in 1972 as an anti-TB drug and has excellent sterilizing activity (Ramaswamy and Musser, 1998). The use of RIF in combination with INH/ PZA led to the establishment of short therapy courses, which reduced routine TB treatment from 1 year to 6 months. RIF is active against both growing and stationary phase bacilli with low metabolic activity. The latter activity is related to its high sterilizing activity in vivo, correlating with its ability to shorten TB treatment. *M. tuberculosis* is susceptible to RIF, with minimum inhibitory concentration (MIC) ranging from 0.05 to 1 µg/ml on solid or liquid

media (Dye and Williams, 2010, Zhang and Yew, 2009). RIF is believed to inhibit bacterial DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits which are α, β, β' and σ, and the genetic loci coding for these subunits include the rpoA, rpoB, rpoC, and rpoD genes, respectively. RIF interferes with RNA synthesis by binding to the β subunit of the RNA polymerase, hindering transcription and thereby killing the organism *(*Dye and Williams, 2010, Herrera *et al.,* 2003).

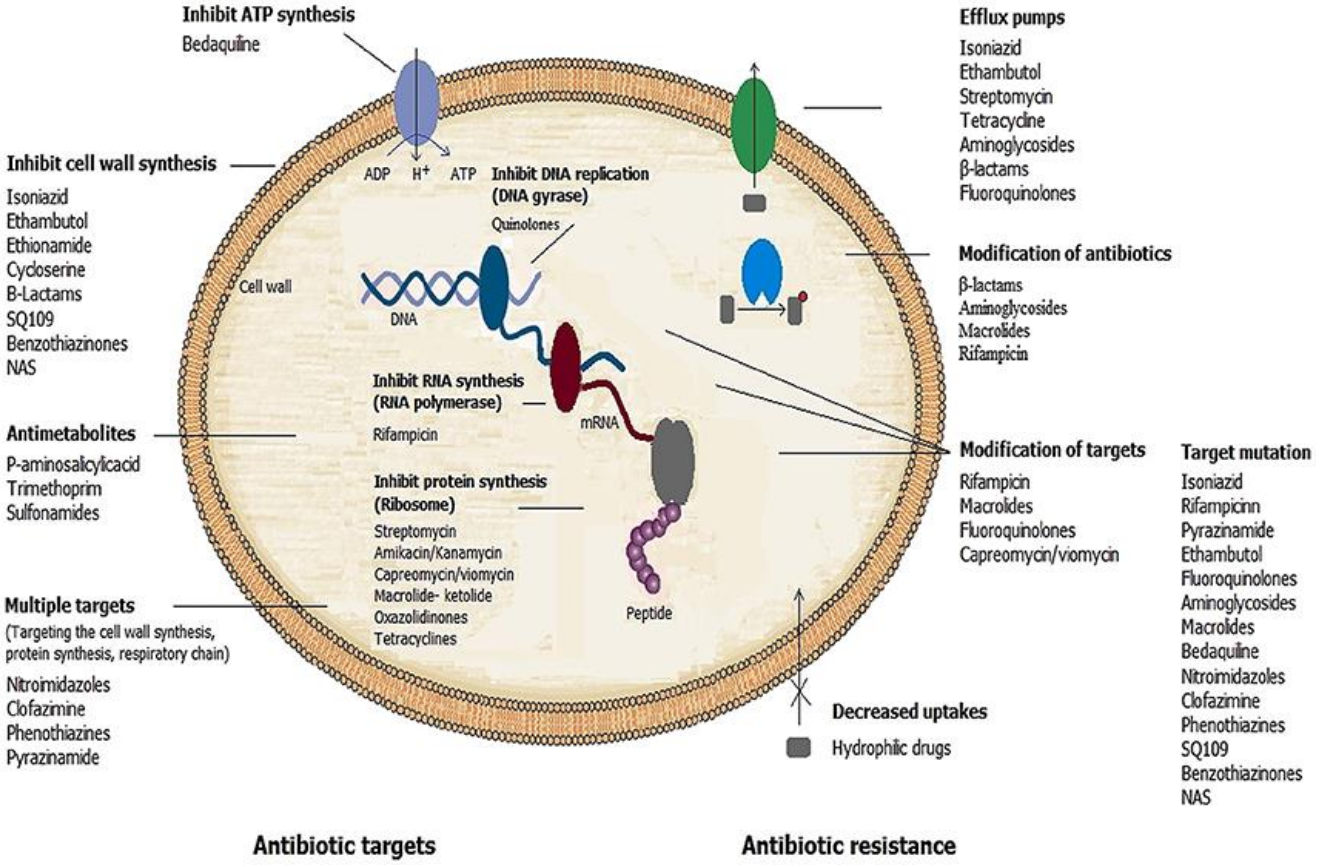


Figure 1-5: Mechanisms of action of antimicrobial drugs and ways by which mycobacteria become resistant to them (Adopted from Nasiri *et al.,* 2017)

Although INH monoresistance is relatively common in MTB, resistance to rifampicin alone is rare, and more than 90% of rifampicin-resistant isolates are also resistant to INH. Resistance to RIF arises due to missense mutations in the gene. In MTB, resistance to RIF occurs at a frequency of 10^{-7} to 10^{-8} (Dye and Williams, 2010). The resistance to RIF develops due to mutations in a distinct, 81-base-pair (bp) (27-codon) central region of the gene that encodes the β-subunit of

RNA polymerase (Herrera L, *et al.,* 2003). Several studies indicated that more than 95% of all mutations are found in the 81-bp core region of the gene between codons 507 and 533, with the most common changes occurring in codons Ser531Leu, His526Tyr, and Asp516Val (Herrera *et al.,* 2003, Rattan *et al.,* 1998, Ramaswamy and Musser, 1998). Mutations in rpoB generally result in high-level resistance (Minimum inhibitory concentration [MIC] > 32 µg/ml) and cross-resistance to all rifamycins. However, specific mutations in codons 511, 516, 518 and 522 are associated with lower-level resistance to rifampin and rifapentine, but retain susceptibility to rifabutin (Zhang and Yew, 2009).

1.10.1.2 Isoniazid (INH)

INH was synthesized in the early 1900's but it is one of the main drugs for the treatment of TB and its latent infections since 1951 (Zhang and Yew, 2009). INH drug mechanism of action is that it penetrates into the cell as a pro-drug activated by katG, the gene encoding catalase-peroxidase (Zhang and Heym 1992). *M. tuberculosis* is highly susceptible to INH (minimum inhibitory concentration 0.02–0.2 µg/ml) (Zhang and Telenti, 2000). The peroxidase activity of this enzyme is essential in activating INH and enabling its interactions with various toxic reactive species in the bacterial cell. The reactive species usually consist of oxides, hydroxyl radicals, and organic moieties, and which reacts with nicotinamide adenine dinucleotide (H), forming INH-NAD adduct that deteriorates components of the cell wall, or causing inhibition of cell wall mycolic acid synthesis (Rozwarski *et al.,* 1998).

INH is the most commonly used anti-TBdrug, resistance to INH occurs more frequently among clinical isolates than resistance to any other agent (Karakousis, 2009). Mutations in INH--resistant clinical isolates are most commonly detected in the katG gene, occurring in 50–80% of cases, thus reducing the ability of the catalase-peroxidase to activate the INH pro-drug (Zhang and Telenti, 2000). The katG gene is located in a highly variable and unstable region of the MTB genome, with missense and nonsense mutations, insertions, deletions, truncation and, more

rarely, full gene deletions observed. InhA enzyme (enoyl-acyl carrier protein reductase), involved in the elongation of fatty acids in mycolic acid synthesis, is considered the primary target of INH inhibition. The replacement of an amino acid in the NADH binding site of InhA apparently results in INH resistance, preventing the inhibition of mycolic acid biosynthesis. A resistance to INH occurs more frequently than for most anti-TBdrugs, at a frequency of 1 in 10^{5-6} bacilli in vitro (Zhang and Yew, 2009).

Various genetic mutations have been observed to occur usually between codons 138 and 328; KatG mutation, where Ser 315 is replaced by Thr (S315T), (Ramaswam and Musser 1998). Mutation in katG S315T is the most common mutation in INH-resistant strains and mutation at Ser315Thr results in an enzyme lacking the ability to activate INH, but preserves approximately 50% of its catalase-peroxidase activity (Zhang and Yew, 2009). Consequently, the modified catalase-peroxidase offers high-level resistance to INH while maintaining a high-level of oxidative protection sufficient to facilitate the organism in sustaining its detoxifying activity against host antibacterial free radicals. Five different nucleotide alterations have been identified in the promoter region of the alkyl hydroperoxide reductase (*ahpC*) gene, which leads to over expression of *ahpC* and INH resistance, *ahpC* overexpression exerts a detoxifying effect on organic peroxides within the cell and protects the bacteria against oxidative damage but does not provide protection against INH. *KatG* expression can also be up-regulated under conditions of oxidative stress (Ramaswamy and Musser, 1998). However the correlation between polymorphic sites in the *ahpC* regulatory region with INH resistance in *M. tuberculosis* requires further studies

Resistance to INH can also occur by mutations in inhA or its promoter region are usually associated with low-level resistance (MICs = 0.2 −1 µg/ml) and are less frequent than katG mutations (Hazbon *et al.,* 2006). As described by Banerjee and collogues mutations in the promoter region of fabG1(mabA)/inhA operon and the InhA active site are causing overexpression of InhA and lowering the InhA affinity to the INH-NAD adduct respectively. INH-resistant MTB

harboring inhA mutations could have additional mutations in katG, conferring higher levels of INH resistance. Mutations in inhA not only cause INH resistance, they also confer cross-resistance to the structurally related drug, ethionamide (Banerjee *et al.,*1994).

Mutations in the promoter region of ahpC in KatG-negative INH-resistant strains, encoding an alkylhydroperoxide reductase, leading to increased expression of the enzyme, have been observed as a compensation for the lack of catalase-peroxidase in such strains. Overexpression of AphC did not appear to confer significant INH resistance (Zhang and Yew, 2009). Up to 25% of low-level INH-resistant strains do not have mutations in katG or inhA, (Hazbon *et al.,* 2006) and it could be due to a new mechanism of resistance. Recently mutations in mshA which encoding an enzyme involved in mycothiol biosynthesis, are shown to confer INH and ETH resistance in MTB strains in vitro however its role in clinical resistance remains to be demonstrated (Vilcheze *et al.,* 2005). Various studies indicated that most of the mutations have been found in katG gene and promoter mutation of the gene inhA was shown to confer low-level resistance to INH. A study conducted in Canada indicated that mutations in 45.4% isoniazid-resistant strains were found in katG315 and 28.6% at inhA promoter region (Bolotin *et al.,* 2009).

1.10.1.3 Pyrazinamide (PZA)

Pyrazinamide, a nicotinamide analog, was first discovered to have anti-TB activity in 1952 and it targets an enzyme involved in fatty-acid synthesis. PZA is one of the important first-line drugs for the treatment of tuberculosis and it plays a unique role in shortening TB treatment from the previous 9–12 months to 6 months. PZA has an excellent sterilizing effect on semi-dormant tubercle bacilli because it kills semi-dormant bacilli in an acidic environment, and minimum inhibitory concentration for PZA is in the range of 16–100 μg/ml (Zhang and Yew, 2015). PZA is a prodrug that is converted to its active form pyrazinoic acid (POA) by the mycobacterial enzyme pyrazinamidase or nicotinamidase, produced intracellularly. The inefficient efflux system of the mycobacterial cell enables massive accumulation of POA in the bacterial cytoplasm, leading to

disruption of the membrane potential (Sheen *et al.,* 2009). The exact mechanism of PZA resistance remains unknown. However, it is known that PZA-resistant bacterial strains usually lose their pyrazinamidase activity and defective pyrazinamidase activity due to pncA mutations is the major cause of PZA resistance (Scorpio and Zhang, 1996).

PZA is active only against MTB complex organisms (MTB, M. africanum and M. microti), but not M. bovis, due to a characteristic mutation in its pncA gene. Strains of M. bovis, including bacille Calmette-Guérin (BCG), are naturally resistant to PZA and lack pyrazinamidase; these features are commonly used to distinguish M. bovis from MTB. The natural PZA resistance in M. bovis and BCG is due to a single point mutation of 'C' to 'G' at nucleotide position 169 of the pncA gene compared with the MTB pncA sequence, causing amino acid substitution at position 57 of the PncA sequence (Scorpio and Zhang, 1996). In MTB, the spontaneous mutations at PZA drug target genes occurs at a frequency of 10^{-6}.

PZA resistance has been attributed primarily to mutations in the pncA gene encoding PZase (Scorpio and Zhang, 1996). Most mutations, including point mutations, deletions, and insertions, have been reported in a 561-bp region of the open reading frame or in an 82-bp region of its putative promoter (Jureen *et al.,* 2008). The pncA mutations are highly diverse and scattered along the gene, which is unique to PZA resistance but there is some degree of clustering at three regions of the PncA, 3–17, 61–85 and 132–142 (Lemaitre *et al.,* 1999). Up to 97% PZA-resistant MTB strains have mutations in pncA, and there are some strains with pyrazinamidase negative and high-level of resistance, it may be due to mutations in an undefined pncA regulatory gene, in addition, such strains have low-level resistance and positive pyrazinamidase activity without pncA mutations; their mechanism of resistance remains to be determined (Lemaitre *et al.,* 1999). A study done in pyrazinamide-resistant strains lacking mutations in pncA showed mutations in *rpsA* (involved on ribosomal protein1) but based on the recent evidence, the contribution of mutations in *rpsA* to pyrazinamide resistance remains limited (Tan *et al.,* 2014).

Table 1-2: Mechanisms of drug action in *M. tuberculosis and responsible gene for* drug resistance adopted from Zhang and Yew (2015)

Drug Name (Year of discovery)	MIC g/ml	Drug Target Gene/ Gene involved in resistance	Gene Function	Role of Gene	Drug Mechanism of Action	Mutation Frequency (%)	Reference
Rifampicin (1966)	0.05–1	rpoB	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis by binding to β subunit of RNA	95	Ramaswamy & Musser, 1998
Isoniazid (1952)	0.02-0.2	katG inhA	Catalase-peroxidase Enoyl acyl carrier protein(ACP) reductase	Drug activation Drug target	inhibition of mycolic acid biosynthesis and other multiple effects	50–95 8–43	Hilleman *et al.,* 2005
Pyrazinamide (1952)	16–100	pncA	Nicotinamidase/ pyrazinamidase	Pro-drug Conversion	Depletion of membrane energy	72–99	Zhang and Yew, 2015
Ethambutol (1961)	1–5	embB	Arabinosyl transferase	Drug target	Inhibition of synthesis of cell wall of arabinogalactan	47–65	Safi *et al.,* 2008
Streptomycin (1943)	2–8	rpsL rrs gidB	S12 ribosomal protein 16S rRNA rRNA methyl transferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis by binding to the 30S subunit of bacterial ribosome	52–59 8–21 Unknown	Zhang and Yew, 2015
Amikacin (1957)	2–4	rrs/eis	16S rRNA	Drug target	Inhibition of protein synthesis	76	Georghiou *et al.,* 2012
Kanamycin (1957)	2–4	rrs/eis	16S rRNA	Drug target	Inhibition of protein synthesis	76	Georghiou et al. 2012
Capreomycin (1960)	2–4	tlyA	2'-O-methyltransferase	Drug target	Inhibition of protein synthesis		Georghiou *et al.,* 2012
Fluoroquinolones (1963)	0.5–2.5	gyrA gyrB	DNA gyrase subunit A DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	75–94	Takiff *et al.,*1994
Ethionamide (1956)	2.5–10	etaA/ethA inhA	Flavin monooxygenase Flavin monooxygenase	Drug activation Drug target	Inhibition of mycolic acid Synthesis	37 56	Zhang and Yew, 2015
Para-aminosalicylic acid (PAS) (1946)	1–8	thyA	Thymidylate synthase	Drug activation	Inhibition of folic acid and iron metabolism?	36	Zhang and Yew, 2015
D- cycloserine (1955)	10–40	*Ddl* *Alr*	D-alanine/D-alanine ligase Alanine racemase,	Drug target	Inhibition of cell wall peptidoglycan synthesis	To be determined	Feng & Barletta, 2003

MIC: minimum inhibitory concentration;

1.10.1.4 Ethambutol (EMB)

Ethambutol is a first-line drug that is used in combination with INH, RIF, and PZA in order to prevent the emergence of drug resistance specific to mycobacterium. EMB is an active bacteriostatic agent for bacilli that are growing but has no effect on non-replicating bacilli. EMB interferes with the mycobacterial cell wall through a synthetic mechanism and inhibits arabinosyl-transferase, which is involved in cell wall biosynthesis (Telenti *et al.,* 1997). The minimum inhibitory concentrations of EMB for *M. tuberculosis* are in the range of 1-5 µg/ml (Zhang and Yew, 2015). Arabinosyl transferase, encoded by embB and involved in the synthesis of arabinogalactan, arabinosyl transferase has been proposed as the target of EMB action within the tuberculosis organism (Lee *et al.,* 2002). In MTB, embB is organized into an operon with embC and embA in the order embCAB; embA, embB and embC share over 65% amino acid identity with each other and are predicted to encode transmembrane proteins (Telenti *et al.,* 1997). Studies showed that the embA and embB proteins are involved in the formation of the proper terminal hexaarabinofuranoside motif during arabinogalactan synthesis (Telenti *et al.,* 1997, Kolyva and Karakousis 2012), while embC is involved in lipoarabinomannan synthesis.

Recently, the most common mutations in embB codon 306 have been reported to be associated with variable degrees of EMB resistance, indicating that such mutations may be necessary but not sufficient for high-level EMB resistance. Other potential mutations involved in EMB resistance include a Gln379Arg substitution in MTB embR, as well as mutations in the rmlD, rmlA2, and Rv0340 genes. As many as one-quarter of all EMB-resistant MTB isolates do not harbor mutations in any of the genes described above, suggesting alternative mechanisms of EMB resistance (Karakousis, 2009). The embB codon 306 mutation is most frequent in clinical isolates resistant to EMB, accounting for as high as 68% resistant strains (Ramaswamy *et al.,* 2000). Studies have shown that resistance to EMB is due to random spontaneous genetic mutations occurring at a rate of approximately 1 in 10^7 organisms; mutations most commonly result in

increased production of the enzyme arabinosyl-transferase, which overwhelms the inhibitory effects of EMB. Studies have identified five mutations in codon 306 including ATG-GTG, ATG-CTG, ATG-ATA, ATG-ATC, and ATG-ATT that result in three different amino acid substitutions (Val, Leu, and Ile) in EMB-resistant strains (Ramaswamy *et al.,* 2000). These mutations are associated with 50–70% of all EMB resistant isolates (Khan *et al.,* 2013, Ramaswamy and Musser, 1998, Ramaswamy *et al.,* 2000, Telenti *et al.,* 1997b).

1.10.1.5 Streptomycin (STR)

Streptomycin (STR) was discovered in 1943 and the first drug used to treat TB. It is an aminoglycoside antibiotic that is active against MTB and a variety of bacterial species. STR kills actively growing tubercle bacilli with minimum inhibitory concentrations of 2–8 μg/ml (Zhang and Yew, 2015), but it is inactive against non-growing or intracellular bacilli (Mitchison 1985). Its mode of action consists of inhibiting the initiation of the translation in the protein synthesis by binding to the 30S subunit of bacterial ribosome, causing misreading of the mRNA message during translation, and the site of action is the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA coded by the genes *rpsL* and *rrs*, respectively (Finken *et al.,* 1993). A study showed that the genetic resistance to streptomycin drug due to spontaneous chromosomal mutations was at a frequency of 2.29×10^{-8} mycobacterial replications (Gillespie 2002).

Resistance to STR is caused by mutations in the S12 protein encoded by rpsL gene and 16S rRNA encoded by rrs gene and the mutations in rpsL and rrs are the major mechanism of STR resistance, accounting for respectively about 50% and 20% of STR-resistant strains. The most common mutation in rpsL is a substitution in codon 43 ((AAG→AGG) from lysine to arginine causing high-level resistance to STR. Mutation in codon 88 is also common and it (AAG→AGG/CAG) (Lys-Arg/Gln) substituting lysine with arginine and glutamine. MIC analysis of STR resistant isolates indicates that amino acid replacements in the *rpsL* genes correlate with a high-level of resistance, whereas mutations in the *rrs* gene correlate with an intermediate level of

resistance (Cooksey *et al.,* 1996). Mutations of the rrs gene occur in the loops of the 16S rRNA and are clustered in two regions around nucleotides 530 and 915. In the *rrs* gene a C-T transition at positions 491, 512 and 516, and A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl–tRNA binding site and is involved in the decoding process. Other mutations in the 915 loop [903 (C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance (Carter *et al.,* 2000).

However, Cooksey and collogues reported that about 20–30% of STR-resistant strains with a low-level of resistance (MIC < 32 µg/ml) do not have mutations in rpsL or rrs, (Cooksey *et al.,* 1996) which indicates that strains resistant to streptomycin that lack mutations in either of these two genes may have other mechanisms of resistance. Recently, a mutation in gidB, encoding a conserved 7-methylguanosine methyltransferase specific for 16S rRNA, has been found to cause low-level STR resistance in 33% of resistant MTB isolates (Okamoto *et al.,* 2007). Another study indicated that while L16R change is a polymorphism not involved in STE resistance, other mutations in gidB appear to be involved in low-level STR resistance. In addition, some low-level STR resistance seems to be caused by increased efflux as efflux pump inhibitors caused increased sensitivity to STR, although the exact mechanism remains to be identified (Spies *et al.,* 2008). Moreover, another finding revealed that the point of mutations in rpsL and rrs genes were found in 52% and 8% of isolates respectively (Heym *et al.,* 1994).

1.10.2 Molecular Mechanisms of Resistance to Second-Line TB Drugs

According to the WHO the following drugs can be classified as second-line drugs: aminoglycosides (kanamycin and amikacin) polypeptides (capreomycin, viomycin and enviomycin), fluoroquinolones (ofloxacin, ciprofloxacin, and gatifloxacin), D-cycloserine and thionamides (ethionamide and prothionamide). Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs (WHO, 2010b). Second-line drugs are mostly used in the treatment of MDR-TB, resulting prolong the total treatment time. The current

understanding of molecular mechanisms associated with resistance and drug mechanisms of action the second-line drugs discussed below.

1.10.2.1 Aminoglycosides (Kanamycin, Amikacin, and Capreomycin)

Kanamycin and its derivative amikacin are a group of aminoglycoside antibiotics, while capreomycin is a cyclic peptide antibiotics group. All drugs are used for the treatment of MDR-TB and they have the same mechanism of action by inhibiting the protein synthesis. Kanamycin and amikacin inhibit the protein synthesis through modification of ribosomal structures at the 16S rRNA (Suzuki *et al.,*1998), and capreomycin bind at the same site in the ribosome at the interface of the small and large subunits (Maus *et al.,* 2005). Mutations at 16S rRNA (rrs) position 1400 are associated with high-level resistance to Kanamycin and Amikacin (Suzuki *et al.,*1998), whereas mutation at a gene of tlyA encoding rRNA methyltransferase was shown to be involved in resistance to CPM (Maus *et al.,* 2005). The minimum inhibitory concentrations of kanamycin, amikacin, and capreomycin for *M. tuberculosis* are in the range in the range of 2–4 μg/ml (Zhang and Yew, 2009).

The most common molecular mechanism of drug resistance has been associated with an A1401G mutation in the rrs gene coding for 16S rRNA. This mutation occurs more frequently in strains with high-level resistance to kanamycin and amikacin. Mutations in tlyA encoding rRNA methyltransferase and the 23S rRNA gene rrs have been implicated in resistance to capreomycin. When a mutation occurs in tlyA gene, there is no rRNA methyltransferase that important for 2′-O-methylation of ribose in rRNA (Johansen *et al.,* 2006). Variable cross-resistance may be observed between KM, and AMK, mutants resistant to CPM could have tlyA, C1402T, or G1484T rrs mutations, and mutation with A1401G could cause resistance to KM and CPM. Moreover isolates resistant to CPM, and KM could have a mutation either a C1402T or a G1484T in the rrs gene. Furthermore, multiple mutations were observed in the rrs gene in a single strain that conferring cross-resistance among these drugs (Maus *et al.,* 2005b). A study done by Jugheli and colleagues

showed that among the 145 isolates sequenced, four types of mutations were observed in the 1400 rrs region: A1401G, C1402T, C1443G, and T1521C and the most frequently observed mutation within the region was an A-to-G substitution at position 1401 (Jugheli *et al.,* 2009)

1.10.2.2 Fluoroquinolones (Moxifloxacin [Mfx] and Levofloxacin [Lfx])

Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB and the mode of action of fluoroquinolones is by inhibiting the topoisomerase II (DNA gyrase). DNA topoisomerases are a diverse set of essential enzymes responsible for maintaining chromosomes in an appropriate topological state and topoisomerases regulate DNA supercoiling and unlink tangled nucleic acid strands to meet replicative and transcriptional needs (Drlica *et al.,* 2003). As Takiff *et al.,* described that in most bacterial species, FQs inhibit DNA gyrase (topoisomerase II) and topoisomerase IV, resulting in microbial death but in MTB, only type II topoisomerase (DNA gyrase) is present and it is the only target of fluoroquinolone activity. DNA gyrase is a tetrameric A2B2 protein. The A subunit carries the breakage-reunion active site, whereas the B subunit promotes adenosine triphosphate hydrolysis and MTB has gyrA and gyrB encoding the A and B subunits respectively (Takiff *et al.,*1994). The minimum inhibitory concentrations of Fluoroquinolones are in the range of 2.5–10 μg/ml (Zhang and Yew, 2009).

The main mechanism of development of fluoroquinolone resistance in MTB is by chromosomal mutations in the quinolone resistance-determining region of gyrA or gyrB. The most frequent mutations found are at position 90 and 94 of gyrA but mutations at position 74, 88 and 91 have also been reported (Sun *et al.,* 2008). A conserved region, the quinolone-resistance-determining region (QRDR) of gyrA (320 bp) and gyrB (375 bp), has been found to be a most important area involved in the exhibition of FQ resistance in MTB (Zhang and Yew, 2009). Mutations within the QRDR of gyrA have been identified in clinical isolates of MTB, largely clustered at codons 90, 91, 94 with Asp94 being relatively frequent. Other major isolates involved also include codons 74, 83, 87 (Sun *et al.,* 2008). A less common involvement is codon 88 and in clinical isolates, gyrB

mutations appear to be of much rarer occurrence. In general, two mutations in gyrA or concomitant mutations in gyrA plus gyrB are required for the development of higher levels of resistance (Zhang and Yew, 2015).

An interesting finding in *MTB* is the presence of a natural polymorphism at position 95 in *gyrA* that is not related to fluoroquinolone resistance since it is also found in fluoroquinolone-susceptible strains and another interesting finding also showed that the simultaneous occurrence of mutations T80A and A90G in *gyrA* led to hyper susceptibility to several quinolones (Palomino and Martin 2014). Lately, a new mechanism of quinolone resistance mediated by MfpA was identified. MfpA is a member of the penta peptide repeat family of proteins from MTB, whose expression causes resistance to FQ drugs. MfpA binds to DNA gyrase and inhibits its activity in the form of a DNA mimicry, which explains its inhibitory effect on DNA gyrase and quinolone resistance (Hegde *et al.,* 2005). The alternative mechanisms co-exist with gyr mutations, the displayed resistance can be anticipated to be considerable. Furthermore, it has been suggested that, with reference to MTB resistance to FQs, the underlying genetic mutations show a substantial disparity among different geographic regions (Zhang and Yew, 2015).

1.10.2.3 Ethionamide

Ethionamide is a derivative of isonicotinic acid structurally similar to isoniazid. It is also a pro-drug requiring activation by a monooxygenase encoded by the ethA gene. It interferes with the mycolic acid synthesis by forming an adduct wit NAD that inhibits the enoyl-ACP reductase enzyme. EthA is regulated by the transcriptional repressor EthR (Carette *et al.,* 2011). Like INH, ETH is also a prodrug that is activated by EtaA/EthA (a monooxygenase) and inhibits the same target as INH, the InhA of the mycolic acid synthesis pathway. The minimum inhibitory concentrations of Ethionamide for *M. tuberculosis* are in the range of 2.5–10 μg/ml (Zhang and Yew, 2009). EtaA or EthA is a flavin adenosine dinucleotide (FAD) containing an enzyme that oxidizes ETH to the corresponding S-oxide, which is further oxidized to 2-ethyl-4-amidopyridine, presumably via the

unstable oxidized sulfinic acid intermediate. In addition, mutations in the target InhA confer resistance to both ETH and INH.

Resistance to ethionamide occurs by mutations in *etaA*/*ethA*, *ethR* and also mutations in *inhA*, which cause resistance to both isoniazid and ethionamide (Brossier *et al.,* 2011). Moreover, studies with spontaneous isoniazid- and ethionamide-resistant mutants of *MTB found* that they map to mshA, encoding an enzyme essential for mycothiol biosynthesis (Vilcheze *et al.,* 2005). Co-resistance to isoniazid and ethionamide can be mediated by mutations that alter the InhA target or cause their overexpression, or by mutations in ndh that increase the intracellular concentration of NADH (Vilcheze *et al.,* 2005). More recently, mshA, a gene encoding a glycosyltransferase involved in mycothiol biosynthesis, has also been suggested as a possible target for ethionamide.

1.10.2.4 Cycloserine

Cycloserine (Cs) is a bacteriostatic drug currently used in the treatment of MDR-TB. It is a cyclic analog of D-alanine which is one of the central molecules of the cross linking step of peptidoglycan assembly (Ramaswamy and Musser, 1998). The minimum inhibitory concentrations of Cycloserine for *M. tuberculosis* are in the range of 1.5–30 µg/ml, depending on the medium of culture used. Cs inhibits cell wall synthesis by competing with D-Alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine racemase (Alr) and also inhibiting the synthesis of these proteins (Ramaswamy and Musser, 1998). Alr is involved in the conversion of L-alanine to D-alanine, which can serve as a substrate for Ddl. The overexpression of alrA encoding D-alanine racemase from M. smegmatis causes resistance to Cs in M. bovis and BCG (Zhang and Yew, 2015). Overexpression of Alr confers higher resistance to Cs than Ddl overexpression in M. smegmatis, suggesting that Alr might be the primary target of Cs. However, a study revealed that the main target of Cs in *M. tuberculosis* is Ddl (Prosser *et al.,* 2013). Zhang and Yew (2015) described that cycA encoding D-serine, L- and D-alanine and glycine transporter involved in the

uptake of Cs was defective in *M. bovis* BCG, it might be related to its natural resistance to Cs. However, the mechanism of Cs resistance in *M. tuberculosis* is still unknown.

1.11. Rational of the study

The emergence and transmission of drug-resistant TB is a major threat and challenge for TB control program (WHO, 2010a), particularly for MDR-TB and XDR-TB, representing a major public health problem globally but more specifically in resource-limited countries. In addition, a high prevalence of TB, poor treatment, limited access to health care, and several other related factors make MDR-TB difficult to address in countries in the sub-Saharan region such as Ethiopia (WHO, 2010b). In the region pattern of MDR-TB and XDR-TB is not well known and little information is available in high TB and HIV prevalence countries such as Ethiopia. Although drug-resistant TB development is a result of unsuccessful TB control programs characterized by inappropriate TB treatment, and poor diagnostic system, there are several risk factors related to drug-resistant TB in this countries.

Ethiopia is one of the 30th high burden countries for TB, TB/HIV and MDR-TB, with TB remaining one of the nation's leading causes of mortality. As reported recently by the WHO, the prevalence of MDR-TB in Ethiopia was estimated to represent14% of previously treated cases, while the prevalence of TB/HIV co-infection was estimated to be 8% (WHO, 2017). However, several studies indicated that the prevalence of MDR-TB higher than WHO estimates with a range between 5%-46.3% (Tessema *et al.,* 2012 Kedir *et al.,* 2015, Dawit *et al.,* 2008, Dereje *et al.,* 2012).

This would appear to indicate that the Ethiopian population could be experiencing a rapid transmission of MDR-TB, that the distribution of MDR-TB varies widely vary from region to region, and that there are significant weaknesses within the nation's TB control program. To address these weaknesses, it was important to generate knowledge about drug resistance pattern and

associated risk factors (WHO, 2010b). The information was very limited in Ethiopia. Therefore, there was a need to study the pattern of the drug-resistant TB and associated risk factors among MDR-TB suspected cases in Addis Ababa which is the epicenter of the socio-economic activity of the country for the development of effective TB control strategies. This study was aimed to identify drug-resistance patterns of *M. tuberculosis* strains among MDR-TB suspected patients and the associated risk factors for the development of MDR-TB in Addis Ababa Ethiopia so as to generate knowledge useful for policymakers with a recommendation for proper management of these patients **(Publication I).**

Drug-resistant (DR) strains of *MTB* arise from spontaneous chromosomal mutations at a predictable low frequency. Studies indicated that selection pressure that is caused by misuse of anti-TB drugs, such as mono-therapy or the addition of single drugs to failing regimens, results in the positive selection of resistant mutants. Drug-resistant TB is occurred due to point of mutations (SNPs) in genes or promoters of genes activating the drug or encoding the drug targets, which are detectable in the majority of drug-resistant isolates (Ong *et al.,* 2010). *M. tuberculosis* isolates could be resistant to RIF, INH, EMB and STR because of mutation in genes and genomic regions. Studies showed that the *M. tuberculosis* becomes resistant to RIF due to the mutations in rpoB, Mutations associated with INH resistance occur mainly in the katG gene, the inhA gene and regulatory region, and the ahpC regulatory region (Piatek, 2000). Mutations associated with EMB occur in embB (Telenti *et al.,* 1997) and pncA gene (Scorpio and Zhang, 1996). However some genes that are responsible for drug resistance are not well known, and a limited information on drug target genes mutation is available in developing countries.

In Ethiopia, although there is high-TB/MDR-TB burden in the population, a limited information is available on the pattern of gene mutation responsible for drug-resistant TB, and associated gene with anti-TB drugs in Ethiopian strains. Moreover, several studies were done in determining drug resistance using different molecular techniques, but there was no Ethiopia-specific data available

on single nucleotides polymorphisms (SNPs) of drug target gene that implicates failure during treatment. Therefore, studying drug target genes mutation responsible for the development of drug resistance TB through sequencing would fill the knowledge gap important to devise TB control program in the metropolis in particular and at large in the country. Thus, the aim of the study was to identify mutations in rpoB, katG, fabG1 (mabA)-inhA, embB, pncA, rrs and rpsL genes associated with the development of resistance to these respective drugs **(Publication II).**

Molecular typing techniques have been extensively used to study strains of *M. tuberculosis* involved in TB infections, studying the molecular epidemiology of *M. tuberculosis*, providing insights into dissemination dynamics, evolutionary genetics, and detection of suspected outbreaks and person-to-person transmission (Brosch *et al.,* 2002). Molecular typing has also contributed significantly to the understanding of TB epidemiology and has helped to improve TB control through providing information on transmission dynamics (Tostmann *et al.,* 2008), and on investigating outbreaks (Paranjothy *et al.,* 2008) including MDR-TB and XDR-TB. Furthermore, molecular typing has revealed that the MTB complexes have a diverse population structure with manifold lineages that show large differences in their geographical occurrence and, also, in their pathobiological properties such as development and spread of drug resistance (Allix *et al.,* 2010).

In Ethiopia, several genotyping studies were done (Mulalem *et al.,* 2010, Amare *et al.,* 2012, Adane *et al.,* 2012, Garedew *et al.,* 2013, Tessema *et al.,* 2013, Mulugeta *et al.,* 2014, Solomon *et al.,* 2015 Fantahun *et al 2015*), however except Tessema *et al.,* Solomon *et al.,* and Fantahun *et al.* studies, all studies were done using spoligotyping technique only which allowed neither for high resolution phylogenetic strain classification nor for analysis of transmission dynamics (Mulalem *et al.,* 2010). As result, a variety of strains were not classifiable into phylogenetic lineages or clonal complexes since the genotyping information was not informative due to homoplasy and the presence of particular genotypes and clustering might simply be overlooked.

(Comas *et al.,* 2009). Thus previous studies had limited by epidemiological information and use of a genotyping method with low strain discriminatory capacity.

Even though those studies done by Tessema *et al.,*, Fantahun *et al.,* and Solomon *et al.,* had conducted using combined spoligotyping and MIRU-VNTR typing method, all of the studies were done in the Amhara Region covering limited geographical area of the country which is different from our study sites and participants type (MDR-TB suspected cases) and socio-economic activities where high influx of people to the metropolis from all corners of the country. So that using of MIRUVNTR 24-loci typing with spoligotyping allows the simultaneous high-resolution discrimination of isolates for epidemiological studies and a stronger phylogenetic strain classification. So analyzing genetic diversity and transmission dynamics of drug-resistant TB isolates would help to determine the genotyping of the circulating strains as well as to track the routes of infection in the population for prevention and control of MDR-TB/TB. Thus, in this study, we investigated the population structure and transmission dynamics of *M. tuberculosis* strains isolated from MDR-TB suspected patients from Addis Ababa using a high resolution MIRU-VNTR 24-loci combined with spoligotyping **(Publication III).**

Moreover, HIV is one of the most important risk factors associated with an increased risk of TB infection progressing to active TB disease. HIV-infected patients have an annual risk of up to 15% of developing active TB once infected. More than 60 % of new cases of PTB patients are co-infected with HIV and found in developing countries in Asia and Africa (Corbett *et al.,* 2003*)*, and TB-related mortality is highest among HIV infected patients, and with HIV-related immunosuppression (Reid and Shah 2009). In addition, diagnosing TB in HIV-infected patients is a major challenge since clinical and radiological findings are often atypical (Lawn and Wood. 2011), with a significant number of smear-negative pulmonary TB patients misdiagnosed. (Davis *et al.,* 2010). A further challenge for TB diagnosis rests with the fact that approximately one-third of TB-HIV co-infected patients fail to produce a sputum specimen for diagnosis of PTB (Peter *et*

al., 2012). As a result, diagnosis TB worse and often delayed among HIV/TB co-infected patients, and as indicated in a study of autopsies that revealed that a large burden of undiagnosed TB was found among HIV-infected patients (Cox *et al.,* 2010), so it is challenging for control of TB at large. From a positive perspective, studies have shown that the rapid diagnosis and initiation of anti-TB treatment can significantly reduce mortality among HIV/TB co-infected patients (Holtz *et al.,* 2011).

However, acquiring a diagnostic specimen remains a major hindrance in HIV-infected sputum scarce patients suspected of having active TB. There is a clear need for new, accurate, and rapid TB diagnostics that have utility in patients who cannot produce sputum. Several studies showed that PTB patients had TB positive urine (Torrea *et al.,* 2005, Gopinath and Singh, 2009, Peter *et al.,* 2012). Moreover, as urine is easily obtainable and noninvasive specimen from sputum scarce cases, evaluation of XpertMTB/RIF molecular method using urine specimen might be a solution. Therefore, the present study was undertaken to evaluate the performance GeneXpert MTB/RIF method using urine specimen from sputum scarce patients an optional method for diagnosis of TB/MDR TB for better control and treatment the disease. **(Publication IV).**

In general, this study is of a paramount importance to the drug resistance TB prevention and control efforts in Ethiopia as it would fill the information gaps that are urgently needed by health care system leaders and health professionals for the proper diagnosis and management of MDR-TB patients, and by TB control program leaders for designing effective prevention and control strategies in the country. Furthermore, researchers in the field of mycobacteriology could benefit out of the findings of this study.Objectives of the Study

1.12. Objectives of the Study

1.12.1 General objectives

The general objective of the study was to generate pertinent data on the patterns of drug resistance, population structure and transmission dynamics of *M. tuberculosis*, and to propose alternative specimen for diagnosis PTB from sputum scarce patients which would be useful for better improvement in diagnosis, treatment, prevention and control of MDR-TB in Ethiopia.

1.12.2 Specific objectives

The Specific objectives of this study were:

- To identify drug-resistance patterns of *M. tuberculosis* strains among MDR-TB suspected patients and the associated risk factors (Publication I).
- To analyze the drug target gene mutations associated with resistance to INH, RIF, PZA, EMB andSTR (Publication II).
- To investigate the population structure and transmission dynamics of *M. tuberculosis* among MDR-TB suspected patients (Publication III).
- To determine the performance of GeneXpert MTB/RIF® Assay using urine specimen for diagnosis of PTB from sputum scarce patients (Publication IV).

1.1. Outline of the thesis

This thesis was divided into seven chapters including the general introduction and each chapter is structured according to the specific objective and journal in which the article is published and if the results in a particular chapter have not been published yet, we have indicated the status of Publication. Therefore the thesis was divided into the following themes which include:

- General Introduction **(chapter 1).**
- Materials and Methods **(chapter 2).**

- Drug-resistance patterns of *M. tuberculosis* strains and associated risk factors among multi-drug-resistant tuberculosis suspected patients from Addis Ababa, Ethiopia **(chapter 3).**
- Analysis of Gene Mutations Associated with Anti-TB Drugs Resistance among *M. tuberculosis* strains Isolated from MDR-TB suspected patients in Addis Ababa, Ethiopia **(chapter 4).**
- Genotyping *M. tuberculosis* Isolates among MDR-TB suspected patients from Addis Ababa, Ethiopia **(chapter 5).**
- Evaluation of GeneXpert MTB/RIF®Molecular Assay using urine specimen for diagnosis of active PTB from sputum scarce patients in Ethiopia **(chapter 6).**
- General Conclusion and Recommendation **(chapter 7).**

In chapter 1: we summarized the general introduction that described the global drug resistance pattern, drug target genes mutations associated with drug-resistant TB, molecular genotyping TB global burden and current TB diagnostic techniques, rational and objective of the study.

In chapter 2: we described the materials and methods part of the thesis. The materials and methods described in details the study setting, study design and population, sample size determination, sampling procedures, specimens and data collection, inclusion and exclusion criteria of study participants, and data processing and analysis as well as ethical consideration. However, we described materials and methods specific to the objective in each corresponding chapter in short summary as per the journal requirments for publication.

In chapter 3: we determined drug-resistance patterns of *M. tuberculosis* strains among MDR-TB suspected patients and the associated risk factors for the development of MDR-TB in the study area. The information would be useful to design effective strategies for control and prevention of MDR-TB in Ethiopia.

In chapter 4: we analyzed the gene mutations associated with resistance to first-line anti-TB drugs such as isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin in *M. tuberculosis* by sequencing. The findings indicated the point of mutations at rpoB, KatG, fabG1-InhA promoter region, pncA, embB, rpsL and rrs genes associated with resistance to rifampicin, isoniazid, pyrazinamide, ethambutol, and streptomycin. The information would be useful to design rapid diagnostic method for diagnosis of MDR-TB.

Chapter 5: described genetic diversity, population structure, and the transmission dynamics *M. tuberculosis* using MIRU-VNTR and Spoligotyping techniques. The isolates were classified taccording to the phylogenetic classification of *M. tuberculosis*. The information helps public health expertise to build sustainable TB/MDR-TB control program that can be useful for diagnosis, treatment, and monitoring of disease progression.

Chapter 6: determined the sensitivity and specificity of GeneXpert MTB/RIF® Assay using urine specimen from sputum scarce patients suspected PTB. This study generated useful information on the performance of GeneXpert MTB/RIF® Assay to urine specimen from the patients could be used as an alternative noninvasive specimen to diagnosis PTB.

Chapter 7: described the conclusion and recommendations. It draws and summarizes the conclusion of the findings from all objectives and it further articulates recommendations and future works based on findings of the study.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Setting

A cross-sectional institution based survey was employed in selected health facilities at Addis Ababa. The study was conducted between June 2015 and December 2016 in Addis Ababa, the capital city of Ethiopia. The estimated population size of Addis Ababa was more than 3.27 million at the time of the study (CSA, 2013). Administratively, the city is divided into 10 sub-cities and further classified into 99 Woreda (lowest government administrative unit). Health facilities which were providing laboratory services for MDR-TB diagnosis and health facilities with high TB patients load were selected from Addis Ababa. Specimen analyses were performed at laboratory facilities of Ethiopia Public Health Institute (EPHI) National Reference TB Laboratory, Ethiopia and Molecular and Experimental Mycobacteriology Research Center Borstel, Germany.

2.2 Study Design and Population

In Ethiopia, drug-resistant TB diagnosis has been carried out using the GeneXpert MTB/RIF assay and phenotypic drug susceptibility testing (DST), however, GeneXpert MTB/RIF assays and DST were being done in few health facilities. Since all MDR-TB suspected cases were referred to GeneXpert MT/RIF diagnostic sites, GeneXpert MT/RIF diagnostic sites found in Addis Ababa City were selected as study sites. Therefore, Addis Ababa Health research and Laboratory services (Addis Ababa Regional referral Laboratory), Teklehiamnot health center, and Saint Peter hospital were the study sites to recruit patients for enrollment in this study. MDR-TB suspected cases reported to these health facilities during the study period were included in the study. MDR-TB suspected case is a patient who is a case of treatment failure, symptomatic patient who had a close contact with confirmed MDR-TB patient; patient from known high-risk group such as health workers; a patient who remains smear positive after 2 months of treatment (new cases) or remains

smear positive after 3 months of retreatment with first-line treatment (retreatment cases such as defaulter, relapse) *(*FMOH, 2009).

For evaluation of the performance of GeneXpert MTB/RIF assay, ten high TB patient load health facilities were selected from each sub city in Addis Ababa. The selected health facilities were Akaki Health center, Arada health center, Zewditu Memorial hospital, Addis Ketema health center, Teklehaimanot health center, Yeka health center, Semen health center, Bole 17 health center, Woreda 9 health center and Kolfe health center. All sputum scarce pulmonary TB (PTB) suspected patients and an equal number of patients capable of giving sputum suspected for PTB who visited the above mentioned health facilities during a study period were included as study participants. Those patients capable of giving sputum suspected of PTB infection were used as a control for the evaluation of the performance of GeneXpert MTB/RIF assay.

2.3 Sample Size Determination

Sample size was calculated using "formula for single population proportion" and a proportion, which could give maximum sample size was chosen.

The required sample size was determined by using single population proportion formula considering the following assumptions: at 95% confidence level (precision) to be 5%.

- Level of significance = 0.05 ♦ Marginal of error (**d**) = 5%
- Proportion/ prevalence = ***P*** ♦Sample size = **n**
- Z (a/2) = Z-score at 95% confidence interval = 1.96

The formula for calculating the sample size (n) was:

$$n = \frac{Z_{\alpha/2}{}^2 P(1-P)}{d^2};$$

According to the 2014 Ethiopian national TB drug resistance surveillance report, the prevalence of drug-resistant TB among previously treated cases was 17.8% (WHO, 2015). *P* = 0.178

$$\mathbf{n} = \frac{\mathbf{1.96^2 x\ 0.178(1-0.178)}}{\mathbf{0.05^2}} = \mathbf{225}, \quad \boldsymbol{n = 225}$$

- There was non-response rate of 10%, the overall sample size(N) was found to be as follow:

N = 225 + 23 (10 % non-response rate) = **248**

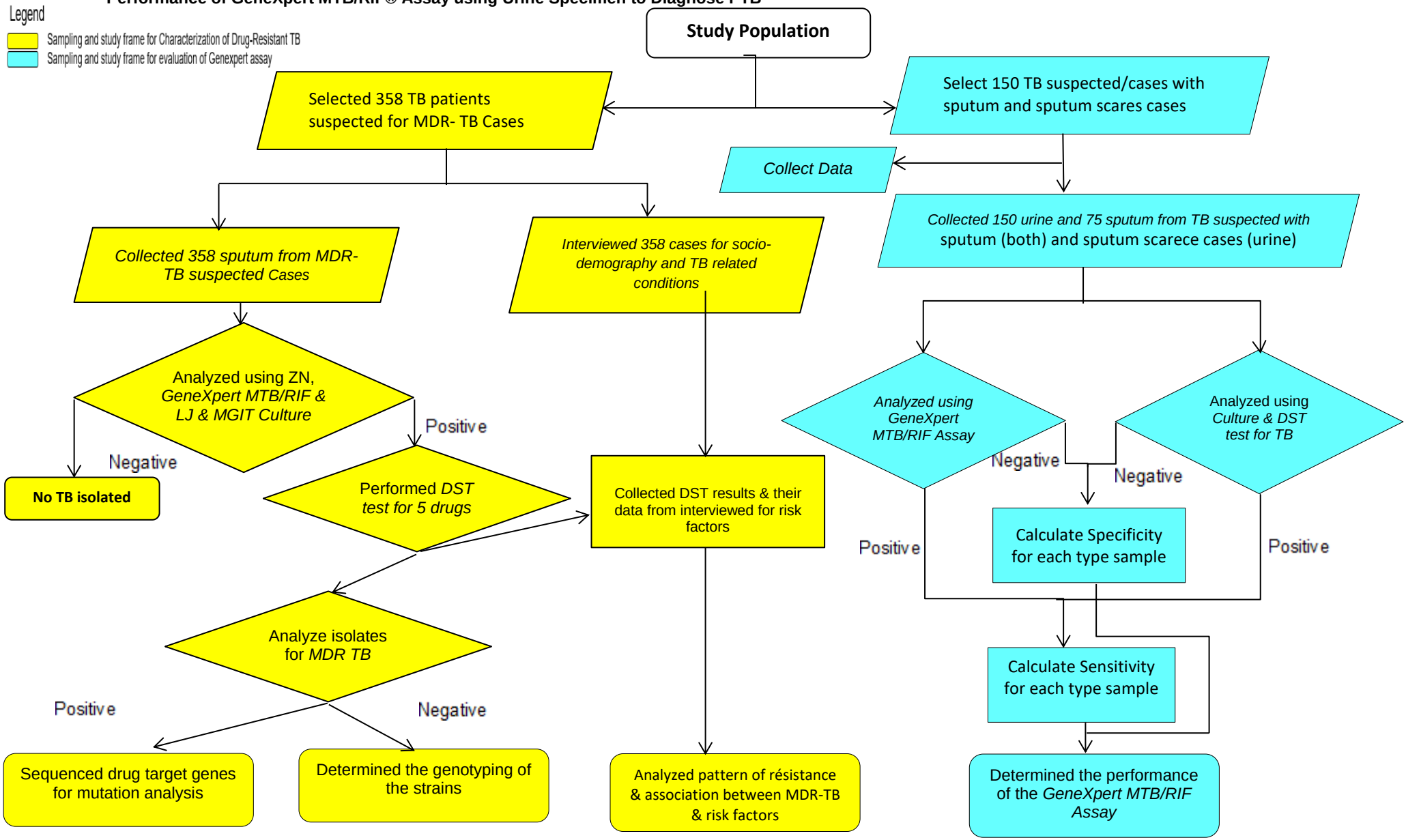
The final sample size for Multi-Drug resistance suspected cases was **248**

For evaluation of the performance of GeneXpert MTB/RIF assay using urine for diagnosis of pulmonary TB (PTB), we included all sputum scarce patients who were suspected for pulmonary TB infection visited the study aerea (health facilities) during the study period. Plus an qual number of patients capable of giving sputum suspected of pulmonary were included for control.

2.4 Sampling Frame

Sputum specimens were collected from all MDR-TB suspected cases, and demographic and TB related data were collected using a questionnaire (Annexed) from patients suspected for MDR-TB for study of the drug resistance pattern and genotyping. For evaluation of the performance of GeneXpert MTB/RIF assay, urine specimens and data (Demographic and TB related information) were collected from sputum scarce patients and patients capable of giving sputum suspected for pulmonary TB infection. In addition, sputum specimens were collected from patients capable of giving sputum reported to study sites (health facilities) during the study period. Both urine and sputum specimens were analyzed using Ziehl-Neelsen (ZN), GeneXpert MTB/RIF assay, culture, and drug sensitivity test (Figure 2.1).

Figure 2-1: Sampling and Study Frame for Study of Molecular Characterization of Drug-Resistant *M. tuberculosis* and Evaluation of the **Performance of GeneXpert MTB/RIF® Assay using Urine Specimen to Diagnose PTB**



2.5 Inclusion and Exclusion Criteria

Health institutions found in Addis Ababa provided MDR-TB diagnosis and TB treatment services were included as study sites. The patients who were suspected of MDR-TB and PTB infection including sputum scarce patients and those capable of giving sputum were part of the study. Those seriously ill/unconscious, below the age of 18, and patients unwilling to participate in the study were excluded.

2.6 Specimens and Data Collection

2.6.1 Specimens Collection

Five to 10 ml sputum specimens were collected using a sterile 50 ml falcon tube according to WHO procedure (1998). A good sputum specimen is considered to be recently-discharged materials from the bronchial tree of the patient, with a minimum amount of oral or nasal materials, and produced by a deep cough of the patients. A morning urine specimen with volume 50 ml was collected using a sterile single collection container (100ml) from sputum scarce patients and patients capable of giving sputum suspected of PTB infection. All the specimens were stored at 2-8˚C to inhibit the growth of unwanted microorganisms at specimen collection sites until transported to EPHI TB laboratory, they were transported using cold chain (2-8˚C), and processed at the same date of collection. The specimen collections were done by trained health professionals.

2.6.2 Data Collection

Questionnaires

Questionnaires were developed based on the objective of the study and required information for the study, and were anonymously closed-ended questionnaire. The questionnaires designed for MDR-TB suspected cases included different questions such as demographic and socio-economic characteristics, educational background, work experience, TB exposure and treatment history,

TB related conditions, alcohol and smoking history and other health related conditions that had an impact in TB. On the other hand, a questionnaire designed for evaluation of the performance of GeneXpert MTB/RIF assay included different questions such as demographic characteristics, work experience, TB history, TB related conditions, and alcohol and smoking history.

Data Collection Procedures

Three senior health professionals for supervision, and 6 health professionals working in TB clinics were involved in the interview. The principal investigator involved in overall controlling activities of data collections. The supervisors assisted data collectors during the process of specimens and data collection and collected filled questionnaires regularly and checked inconsistencies and omissions, and the integrity of specimens, storage and transportation system. These data collectors and supervisors were trained for two days, including both theoretical and practical training. The training was based on the guide that was adopted from standards by the principal investigator for data collectors and supervisors to clarify how to administer the questionnaire and collect and handle the specimens. Besides, the data collectors were trained on their responsibility for describing the purpose of the study, giving orientation, informing patients about the importance of honest and sincere reply on responding to questions.

During the data collection period, the data collectors explained the study objectives and take oral and or informed consents from study participants. Accordingly, the data collectors conducted the interview for those patients who were willing to participate in the study. Laboratory result and secondary data were collected from TB and MDR-TB registers found at each site. Questionaire was developed in English and translated into Amharic then back to English to check for its consistency. Pre-testing of the questionnaire was done on establishments which were not included in the actual study in order to check its language clarity and consistency of the questionnaire. An ambiguous part of questionaire was corrected after pretesting and communicated with data collectors.

Eligibility and Recruitment of Study Participants

MDR-TB suspected patients and pulmonary TB suspected patients including sputum scarce patients and those capable of giving sputum were eligible for study particpants. MDR-TB suspected patient was defined according to FMOH (2009): a patient who is a case of treatment failure; a symptomatic patient who had a close contact with confirmed MDR-TB patient; a patient from known high-risk group such as health workers; a patient who remains smear positive after 2 months of treatment (new cases); or remains smear positive after 3 months of retreatment with first-line treatment (retreatment cases such as defaulter, relapse). Moreover, pulmonary TB suspect patient is any person who presents with symptoms of TB involving the lung parenchyma that the common symptom of pulmonary TB is a productive cough for more than 2 weeks, 1 which may be accompanied by other respiratory symptoms (shortness of breath, chest pains, haemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats, and fatigue). During the study period the eligible MDR-TB suspected and pulmonary TB suspected patients and voluntary to participate in the study was recruited, and patients who were willing to participate in the study gave verbal or written consent at each health facility.

2.7 Laboratory Analysis

2.7.1 Ziehl-Neelsen/Acid Fast Bacilli Staining (AFB) Technique

The Ziehl-Neelsen acid-fast staining was used based on the method described by WHO (1998). Briefly, a smear was prepared using a clean slide from yellowish mucopurulent part of the sputum. The sputum was spread on the slide evenly by smearing repeatedly in a coil. The smear was dried at room temp and fixed by passing the slide over the flame 2-3 times. The smear was placed upwards on staining bridge and covered with filtered 1% carbol fuchsin solution, and heated until steam rises from the stain and then left for 5 minutes. After 5 minutes, the slide was washed with running water to remove the staining solution and made tilted to drain off excess rinse water. The 3% Acid Alcohol solution was added to the slide for decolorizing carbol fuchsin solution and after

3 minutes the solution was washed with running water. The 0.3% methylene blue solution was added on the slide and washed with running water after 1 minute, finally, the slide was placed upright on the slide rack to dry.

The stained slide was examined using a 100× oil immersion objective on a light microscope by horizontal scanning manner for at least 100 visual fields to determine the result. Acid fast bacilli stain pink, straight or slightly curved rods and the background appears blue due to methylene blue. Result Interpretation, if no bacilli found in at least 100 fields, the result was reported as negative. If bacilli was found, the result was reported as positive with semi-qualitative way such as 1 to 9 bacilli per 100 fields reported as scanty, 10 to 99 AFB per 100 fields reported as 1+, 1 to 10 bacilli per field (count at least 50 fields) reported as 2+, more than 10 bacilli per field reported as 3+.

2.7.2 GeneXpert MTB/RIF Analysis using Sputum and Urine Specimens

The GeneXpert MTB/RIF assay is a hemi-nested real-time PCR method that amplifies the 81-bp region of the RIF-resistance determining region of the rpoB gene, positions 507–533. A sputum sample reagent buffer containing NaOH and isopropanol was mixed in a 2:1 ratio to the processed sputum ensuring a final volume of at least 2 ml, and then incubated 10 minutes. Again the specimen was shaken vigorously for 10 times and afterward incubated at room temperature for 5 minutes (Barnard *et al.,* 2012). For urine specimen, the specimen was centrifuged at 3000 *g* for 15 minutes and the pellet re-suspended in 1 ml of sterile phosphate-buffered saline. The sample reagent was mixed at a 2:1 ratio with 1 ml of re-suspended urine and the suspension was shaken vigorously for 10–20 times (Peter *et al.,* 2011). The specimen was incubated at room temperature for 10 minutes. Again the specimen was shaken vigorously for 10–20 times and afterward incubated at room temperature for 5 min.

After final incubation, 2 ml of the liquefied inactivated specimen was added to the cartridge that contains the wash buffer, reagents for lyophilized DNA extraction and PCR amplification, and fluorescent detection probes (five for the rpoB gene and one for an internal control, Bacillus globigii spores). After the cartridge was placed in the instrument module, the automated processes included the following: specimen filtering, sonication to lyse the bacilli and internal control spores, released DNA collection and combination with the PCR reagents, amplification, target detection by five-color fluorescence of overlapping molecular beacon probes, and one color fluorescence for the internal control. Finally, results were automatically generated within 2 hours and reported as MTB-negative or positive and RIF sensitive or resistant (Barnard *et al.,* 2012, Theron *et al.,* 2011).

2.7.3 Culture of *M. tuberculosis*

Sputum and urine specimens were cultured on Lowenstein-Jensen (LJ) solid media and MGIT after decontamination. The egg-based culture medium Löwenstein-Jensen was prepared as described by WHO (1998). Briefly, a culture medium was prepared by dissolving the salts (2.4g of Potassium dihydrogen phosphate anhydrous (KH_2PO_4), 0.24g of Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.6g of Magnesium citrate, 7.2g of Sodium glutamate,) in 600ml of distilled water, and adding of 12ml of glycerol and 20ml of 2% Malachite green solution, and sterilized the solution by autoclaving at 121°C for 30 minutes. Then mixed the sterilized solution (600ml) with homogenized eggs (1000ml) in a beaker. Finally, quality control was done by incubating the media for 2 days at 37 °C to check for sterility.

Five 5 ml of sputum purulent part was transferred to another falcon tube and added equal volumes of NALC-NaOH solution. Using aliquots of NALC-NaOH (1 vial of NALC-NaOH per one specimen) and tightened cap of container and vortex slowly and inverted each bottle to ensure that NALC-NaOH solution contacts the inner portion of caps. Kept at 20°C – 25°C for 15 minutes for decontamination, and filled the tube with phosphate buffer up to 45 ml mark on the tube using

aliquots of phosphate buffer (1 vial of PBS per one specimen). Mixed-well using vortex and centrifuged at 3,000 ×g for 15 minutes, and then the discarded supernatant, and re-suspended the deposit with 2ml PBS.

Labeled each LJ tube with specimen number and decanted excess water from the media, and inoculated 2 to 3 drops of sediment into two LJ media, and loosen the cap slightly and kept in a slant position for one week and checked LJ two times a week for any contamination and fast grower Mycobacterium /NTM. Then read LJ tubes weekly until a positive growth obtained. If the LJ tube has growth, the colonies were buff colored (never yellow) and rough, having sometimes the appearance of bread crumbs or cauliflower. However, the morphology of the colonies varies if specimen was very old, or specimens obtained from patients while on treatment. Microscopically they were frequently arranged in serpentine cords of varying length or show district linear clumping with 3µm to 4µm in length (WHO, 1998). The culture testing results were recorded as 3+ for confluent growth; 2+ for more than 100 colonies; 1+ for 20-100 colonies, and < 20 colonies for the actual number of colonies. If there is no any growth in the 8^{th} week, reported as negative

For MGIT, labeled each MGIT tube with specimen number and added 0.8 ml of the PANTA solution using repeater pipette to each MGIT tube then added 0.5 ml of a well-mixed processed and/or direct specimen to the appropriately labeled MGIT tube using disposable Pasteur pipette. Tightly recapped the tube and mix by inverting the tube several times and left at room temperature for 30 minutes. Finally, the MGIT tube was incubated in MGIT 960 instrument according to manufacturer instruction. Checked MGIT 960 daily for indicator lights flagging positive and negative cultures and Incubated MGIT tubes until the instrument flags them as positive (red flag) or negative (green flag).

2.8.4 Identification of *M. tuberculosis*

Results obtained from on LJ and MGIT were identified on the basis of colony morphology, growth rate, pigmentation and properties. However, all positive culture results were confirmed by using MPT64 antigen detection methods (Capilia)**.** The test device consisted of a sample area, a test area containing the anti-MPB64 antibodies, and a control area where anti-species immunoglobulin antibodies are fixed. The testing method is based on immunochromatographic principles, in which antibodies labeled with colloidal particles react with target antigens to form a migrating antigen-antibody complex, which is captured by a second fixed antibody. A color reaction takes place where the labeled particles are fixed (ECDC, 2016). The test was performed as per manufacture procedure (Capilia TB-neo Becton, Dickinson Diagnostic Systems, Sparks, MD, USA)

Test device was labeled with specimen number and added specimen into the test device sample area, a 100 µl volume from MGIT/ liquid cultures or , from solid cultures, 1 µl bacteria (=1 mm loop) re-suspended in the respective buffer, then vortexed and a 100 µl volume of the suspensions was added to test device and incubated 15 minutes. After 15 minutes, the result is interpreted as positive for *M. tuberculosis* complex if the color reaction takes place in the test and control area. If there was no any color reaction in test area but in presence of a color band in control area, the specimen was interpreted as negative.

2.8.5 Drug Susceptibility Test (DST)

Phenotypic drug susceptibility test for rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) were performed with the Bactec MGIT 960 method. Briefly, 0.5 ml of a bacterial suspension was inoculated into each vial supplemented with reconstitution solution, and a particular drug, such as 1.0 µg/ml for RIF, 0.1 µg/ml for INH, 5.0 µg/ml for EMB 1.0 µg/ml for STR and 100 µg/ml for PZA and 0.1 ml of a bacterial suspension (1:100 dilution) was inoculated into a vial of Growth Control (GC) supplemented with reconstitution

solution only (Siddiqi *et al.,* 2006). *M. tuberculosis* strain H37Rv (ATCC 27294) was used as a susceptible control for the susceptibility testing. Detail procedures desribed below.

2.8.5.3 DST using MGIT Method

Susceptibility testing in the MGIT 960 system is based on the same principles as isolation from sputum (detection of growth). DST is performed using an AST (antibiotic susceptibility testing) set or TB exit, which consists of a Growth Control tube and one tube for each drug. A known concentration of a drug is added to a MGIT tube, along with the specimen, and growth is compared with a drug-free control of the same specimen. If the drug is active against the mycobacterial isolate (isolate susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube; meanwhile, the drug-free control will grow and show increasing fluorescence. If the isolate is resistant, growth and its corresponding increase in fluorescence will be evident in both the drug-containing and the drug-free tube. The MGIT 960 system monitors these growth patterns and can automatically interpret results as susceptible or resistant. An isolate is defined as resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug. The detailed procedure for MGIT DST test was as follow:

2.8.4.3 Inoculum preparation for DST Using MGIT

Each lyophilized drug vials of Streptomycin, Isoniazid, Rifampicin and Ethambutol (SIRE) was reconstituted with 4 ml of sterile distilled water, and PZA drug vial was reconstituted with 2.5 ml of sterile distilled water. Pure 1-2 days old MGIT positive culture tube, was vortexed for 1 minute to break up clumps and left undisturbed for about 20 minutes to allow large clumps to settle. For a culture with 3- 5 days old, dilution 1:5 (1.0 ml of the positive broth and in 4.0 ml of sterile saline) was made after vortex for 1 minute. For inoculation, 1:100 was prepared from the culture (1-2 days old tube, or 1:5 diluted of 3-5 days old tube) for Growth Control (GC) by adding 100 µl of the

culture into 9.9 ml of saline, and for DST 1:10 was prepared by adding 500 µL of the culture into 4.5 ml saline.

2.8.4.4 Inoculation into SIRE set

Five MGIT tubes were labeled for each test isolate with GC (Growth Control), STR, INH, RIF and EMB and specimen number. Aseptically 0.8 ml MGIT SIRE Supplement was added to each SIRE tube including growth control tube. Then aseptically 100 µl of each reconstituted drug solution was added into the corresponding labeled MGIT tubes, and no drug was added into GC tube. Aseptically 0.5 ml of specimens from 1:100 diluted tube was dispensed into the SIRE GC tube using a sterile transfer pipette. Each labeled drug-containing tube was inoculated with 0.5 ml of 1:10 diluted culture by aseptic technique just putting it inside the mouth of the tube so that the inoculum runs down the inside of the tube. The tubes were immediately tightly recapped and mixed by inverting twice. Finally, all tubes were disinfected with a mycobactericidal disinfectant.

2.8.4.5 Inoculation into PZA set

Two PZA tubes were labeled for each test isolate with GC (Growth Control), or PZA, and specimen number. Aseptically 0.8 ml of PZA supplement was added to each PZA tube. Then, 100 µl of the reconstituted PZA drug solution was added into the corresponding labeled MGIT tubes, no drug was added to GC tube. Aseptically 0.5 ml of the 1:10 diluted culture tube was dispensed into the PZA GC tube using a sterile transfer pipette. PZA drug-containing tube was inoculated with 0.5 ml of the Inoculum. The tube was immediately tightly recapped and mixed by inverting twice. All tubes were disinfected with a mycobactericidal disinfectant. Finally, entered the inoculated set of DST specimens into the BACTEC 960 instrument according to manufacturer instruction (The BD BACTEC™ MGIT™ 960 system) using the AST set entry feature. Checked MGIT 960 daily for indicator lights flagging positive.

2.8.4.6 DST Result Interpretation from MGIT

The MGIT 960 system automatically interprets the results of the test based on the growth unit (GU). If the GU is greater than 100 for a drug-containing MGIT bottle, the isolate is resistant ("**R**") and if the GU is less than or equal to 100 then the isolate is susceptible ("**S**"). However, for the test to be valid, the GU of the control bottle cannot reach 400 before 4 days or after 13 days, which suggests that the growth was too heavy or too light respectively. An isolate was defined as **Resistant**: if 1% or more of the test population grows in the presence of the critical concentration of the drug. An isolate was defined as **Susceptible**: if there was no growth or less than 1% of the test population grows in the presence of the critical concentration of the drug.

2.8.5 Genomic DNA Extraction for Sequencing and Genotyping Analyses

Genomic DNA of Mycobacterium was extracted from the culture of *M. tuberculosis* colonies by a method described by Somerville *et al.* (2005). The procedure was as follow: A loop full of *Mycobacterial tuberculosis* colonies from culture was suspended in 400 µl of 10 mM Tris–HCl, 1 mM EDTA with pH 8 buffer. Then it was heated for 20 minutes at 80°C for killing all mycobacterium tuberculosis. Then 50 µl lysozyme (10 mg/ml) was added, and incubated for 2 hours at 37°C. After incubated for 2 hours at 37°C, 75 µl mix of 5 µl proteinase K (10 mg/ml) and 70 µl 10% sodium dodecyl sulfate (SDS) was added into the mixture, and tubes were vortexed and incubated at 65°C for 20 minutes with continuous shaking. After incubation, 100 µl mixture of N-acetyl-N, N, N-trimethyl ammonium bromide (CTAB) [40 mM] and NaCl (0.1 M) was added, and then added NaCl (final concentration: 0.6 M) immediately. And the mix vortexed until the suspension turned milky, and incubated at 65°C for 10 minutes. After incubated at 65°C for 10 minutes, 750 µl chloroform-isoamyl alcohol (24:1) was added, and again vortexed. And then centrifuged at 13,000 rpm in a microcentrifuge for 5 minutes at room temperature. The genomic DNA was then precipitated with 70% ethanol and re-suspended in 30 µl TE buffer. Finally, DNA quality and concentration were determined by spectrophotometer at a optical density of 260 nm and 280 nm.

The ratio of 260/280 nm was used to determine the quality of DNA. Most of DNA specimens should give ratio between 1.5 and 2.0. The specimens that had lower ratio was retreated with chloform-isoamyl alcohol to remove proteins, and repeated the previou procedures.

2.8.6 PCR amplification for Sequencing

The PCR amplification of the first line drug target genes of *M. tuberculosis* were done by using gene specific primers, the details presented in table 2.1. These primers were also used during sequencing. The PCR reactions were conducted in volume of 25 µL final reaction mix containing: 2.5 µl of 10x PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.001% gelatin), 0.5 µl of 0.2 mM dNTPs, 0.75 µl of 25 mM magnesium chloride solution, 1.25 µl each of the 10 µM primers (forward and reverse primers), 0.125 µl of 2 U AmpliTag Gold polymerase (Perkin Elmer, USA), 1.25 µl of DMSO (Dimethylsulfoxide) 2 µl of genomic DNA, and 15.3 µl of double distilled water. The amplification was done by programming the thermocycler of Eppendorf™ up on the following condition: 95°C for 3 minutes for initial denaturation; followed by 40 cycles of denaturation at 95°C for 1 minute, annealing ranges from 55°C to 65°C for 30 second or 1 minute (see table 2.1 for each gene), and extension at 72°C for 30 seconds. The final extension was at 72°C for 5 minutes. The PCR amplified products were examined on 1.5 % agarose gel electrophoresis using 100 base pairs DNA ladder

Then, EXOSAP cleanup of PCR products for sequencing was performed up on the following condition: 5 µl PCR products were mixed with 1µl exonuclease and 1µl alkaline phosphatase and then the mix was then placed in a thermal cycler with a hot lid off. The cycles were performed for 30 min at 37°C and 15 min at 80°C. After EXOSAP cleanup, the PCR products were sequenced with their gene specific forward and reverse primers using Big dye- terminator kit as follow: 1 µl EXOSAO cleaned up PCR products were mixed with 1.5µl sequence buffer, 0.25 µl of the required primer, and 6.25 µl of double distilled water. For gene that used more than one primers, separate mix sequence-PCR was prepared for each primer. Then the mix was then placed in thermal

cycler. The cycles were performed for 15 seconds at 96°C and 90 seconds at 60°C for 24 cycles for each (Khan *et al.,* 2013).

Finally, Sephadex cleanup of sequence-PCR products was performed as follow: 300 µl of double distilled water was added for each sequence-PCR product and incubated for 1 hour at room temperature, and then centrifuged at 920 rpm (revolution per minute) for 5 minutes. Added 15 µl of formamide to new ABI-plate and then 4 µl Sephadex cleanup of sequence-PCR product, and closed it. Finally vortexed and centrifuged, and closed with sequencing lid. It is read for sequencing.

Table 2-1: Primers that were used for PCR amplification and DNA sequencing of drug target genes for analysis of mutation in Mycobacterium isolates.

Gene		Primer Sequence (5'→3')	Position	Annealing	Amplicon Size (bp)	Reference
rpoB	Forward	TCGCCGCGATCAAGGAGT	981	65°C (30 sec)	272	Homolka *et al.,* 2010
	Reverse	GTGCACGTCGCGGACCTCCA	1253			
katG	Forward	TCGGCGATGAGCGTTACAGC	614	65°C (30 sec)	542	Zhao *et al.,* 2005
	Reverse	CCCGCAGCGAGAGGTCAGTGG	1156			
fabG1-inhA	Forward	CCTCGCTGCCCAGAAAGGGA	-168	55°C (1 min)	229	Talenti *et al.,* 1997
	Reverse	ATCCCCCGGTTTCCTCCGGT	61			
rpsL	Forward	CGGCGGGTATTGTGGTTGCTCGTG	-203	55°C (1 min)	800	Khan *et al.,* 2013
	Reverse	CCTCCAGGGCGGGTTTGACATTG	597			
Rrs	Forward	CTTATGTCCAGGGCTTCA	1202	55°C (1 min)	363	Khan *et al.,* 2013
	Reverse	CAGTTGGGGCGTTTTC	1565			
embB	Forward	TGGACGGGCGGGGCTCAAT	725	65°C (30 sec)	575	Khan *et al.,* 2013
	Reverse	CCAGCGCCGCCGGTGTGAGC	1300			
pncA	Forward	GCTGGTCATGTTCGCGATCG	-104	60 °C (30 sec)	708	Koivula *et al.,* 2004
	Reverse	CGCTCCACCGCCGCCAACAG	604			

2.8.7 DNA sequencing and analysis

The resulting products that sequenced with their gene specific forward and reverse primers using Big dye- terminator kit and ABI Prism 3500lL Genetic Analyzer (Applied Biosystems, USA). The sequencing data obtained from the ABI 3730XL DNA analyzer were imported into SeqScape softwwere version 2.7 (Applied Biosystems, Foster City, CA) and consensus sequences

generated. The SeqScape was used for DNA sequence comparisons and mutations were detected in a respective gene by comparing with the reference *M. tuberculosis* strain H37Rv sequence.

2.8.8 PCR amplification and analysis for Genotyping

2.8.8.3 Spoligotyping

Spoligotyping was performed for 204 *M. tuberculosis* isolates, using the standard method, to detect the presence or absence of 43 spacers (Kamerbeek *et al.,* 1997). Briefly, the DR region was amplified from 2 µL of mycobacterial DNA (10 ng) in 20 µL of a reaction mixture containing: 10 µl of Master Mix (2 µl of 10x PCR buffer, 0.5 µl of DMSO, 0.05 µl of 100 mM dNTPs, 0.6 µl of 50 mM magnesium chloride solution, 1 µl of the 10 µM primer DRa and 1 µl of the 10 µM primer Dra, 0.1 µl of Taq Polymerase and 12.8 µl of double distilled water, according to manufacturer's instructions (Ocimum Biosolutions, India). The amplification was done by programing the thermocycler of Eppendorf™ up on the following condition: 5 minutes at 95˚C and subjected to 30 cycles for 1 minute at 94˚C, 1 minute at 58˚C, 30 seconds at 72˚C, 5 minutes at 72˚C, and kept at 4˚C untill hybridization performed.

After DNA amplification, 20 µl of PCR products was added to 150 µl 2x SSPE + 0.1% SDS and heated denature the diluted PCR products for 10 minutes at 100°C and cool on ice immediately. After washing the membrane for 5 minutes at 60°C in 250 ml 2x SSPE/0.1% SDS once. Each sample was loaded on the blotter with a membrane laid on it and incubated at 65˚C for 1 hour. The membrane was removed from the blotter washed in SSPE buffer on a shaker at 60˚C for 15 minutes. PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to the spoligo-membrane (Isogen Life Sciences, The Netherlands) according to the manufacturer's instructions. Bound fragments were detected by chemiluminescence after incubation with a streptavidin-peroxidase conjugate (Boehringer Ingelheim, Germany) and assessed by an enhanced chemiluminescence system (GE Healthcwere UK Limited,

Buckinghamshire, UK). Spoligotypes were reported using an octal code in which the 43-digit binary representing the 43 spacers ("1" is hybridization and "0" is no hybridization) was divided into 14 sets of three digits (spacers 1-42) plus one additional digit (spacer 43). Each three-digit set was converted to octal code (000 = 0, 001 = 1, 010 = 2, 011 = 3, 100 = 4, 101 = 5, 110 = 6 and 111 = 7), with the final digit remaining either 1 or 0, yielding a 15-digit octal designation (Dale *et al.,* 2001).

2.8.8.4 MIRU-VNTR typing

A total of 204 isolates were genotyped using 24-loci MIRU-VNTR genotyping technique in the Borstol Research center Germany, as described previously by Supply *et al.,* (2006). Briefly, 24 loci were amplified by using the MIRU-VNTR typing kit (Genoscreen, Lille, France), and the PCR products were analyzed using the Rox-labeled MapMarker 1,000 size standard for mix 1–4, and mix 6–8, and 1500 size standard for mix 5 (BioVentures, Inc., Murfreesboro, VT) by ABI 3730XL sequencer with 16 capillaries (Applied Biosystems, Foster City, CA). Analysis of the PCR fragments and assignment of the various VNTR alleles were done using the GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA).

PCR mixtures were prepared as follows, using 96-well plates and the Hot StartTaq DNA polymerase kit (QIAGEN, Hilden, Germany). Two nano grams of DNA was added to a final volume of 20 μl containing 0.08 μl of DNA polymerase (0.4 U); 4 μl of Q-solution; 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden); 2 μl of PCR buffer; 1.5 to 3.0 mM MgCl2; 0.4 μM of each unlabeled oligonucleotide; and from 0.04 to 0.4 μM of labeled oligonucleotide. The Multiplex PCR kit (QIAGEN, Hilden, Germany) was specifically used for mix 5 to avoid pronounced stutter peaks observed with large alleles of QUB-26. Therefore, 2 ng of DNA was added to a final volume of 20 μl containing 10 μl of PCR Master Mix; 1 μl dimethyl sulfoxide; and

0.08, 0.28, and 1 μM of each unlabeled and labeled oligonucleotide for loci 2163a, 1955, and QUB-26, respectively (Supply *et al.,* 2006).

2.8.8.5 Interpretation of genotyping results

The observed spoligotypes were compared to the international database, SITVIT, which is an updated version of the published SpolDB4.0 database (Brudey *et al.,* 2006) and it is available at pasteur-guadeloupe.fr:8081/SITVITDemo/. For previously unreported spoligopatterns in the Spol-DB4.0, the "Spotclust" database was used. This model takes into account knowledge of the evolution of the DR region and assigns spoligopatterns to families and subfamilies, using a computer algorithm based on studies of SpolDB3 (cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html). BioNumerics softwwere (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of spoligotyping and MIRU-VNTR patterns. Dendrograms were constructed for spoligotyping, MIRU-VNTR and the combination of both methodology, it was generated by using the unweighted pair group method with arithmetic averages (UPGMA).

The cluster analyses were carried out by the Bionumerics software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium) using the manufacturers' instructions, and similarities of genotyping patterns of strains were calculated by using the categorical coefficient. In addition, minimum spanning tree analysis was carried out based on MIRU-VNTR data by using the categorical coefficient. For the analysis of cluster, a cluster was defined as a minimum of two strains sharing identical genotype pattern using combined data of MIRU-VNTR 24-loci and spoligotyping from the study subjects. Moreover, recent transmission index (RTI) was calculated using the formulas described as follow: **[number of clustered patients - number of clusters]/total number of patients**.

2.8.9 Quality Assurance for Specimens Analysis

To assure quality of laboratory analysis, specimens and data collectors were senior health professionals and trained for two days on specimens handling, transportation and storage including safety practices. All specimens were transported and stored according to the requirement for sputum and urine specimens handing procedure, and processed at the same date of collection. Culture, DST, GeneXpert assay and AFB analyses were performed at Ethiopia Public Health Institute, National TB Reference Laboratory which is quality assured and accredited laboratory by ISO 15189: 2012, Medical laboratories — Requirements for Quality and Competence. Moreover, sequencing, Spoligotyping and 24-loci MIRU-VNTR genotyping analysis were performed at the Molecular and Experimental Mycobacteriology Research Center Borstel, Germany, which is a European WHO TB Supranational Reference Laboratory. All analyses were performed by senior and experienced laboratory professionals and during the analysis were performed using calibrated instruments and quality controls were performed according to the methods requirements. All ambiguous analysis output were repeated for better and accurate results.

2.9 Study Variables

The main variables included in the study instrument were classified as dependent and independent variables. The dependent variable measured was acquired MDR-TB. The independent variables measured were demographic data, socioeconomic status, occupation, educational status, HIV status, adherence status to the first course of anti-TB treatment, number of previous anti-TB treatments, ever- interruption in taking medicine for a day, occurrence of drug side effects during the first course of TB treatment, alcohol drinking history, smoking history, MTB strains genotyping, gene mutation in the MDR TB isolates.

2.10 Data Processing and Analysis

2.10.1 Data Analysis for Determination of Risk Factors for Development of DR-TB

Data were entered using Microsoft Excel and exported to SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Data completeness and consistency were checked by running frequencies of each variable. Descriptive statistics were computed for all collected variables. Bivariate analyses was carried out for categorical variables, and odds ratios were used to quantify the strength of association between potential risk factors and MDR-TB. Multiple logistic regressions was used to control the confounding effect of different variables while assessing the effect of each variable on the likelihood of MDR-TB occurrence. A p-value of 0.05 was used as the cut-off point for statistical significance. Variables having a p-value of at most 0.05 in bivariate analysis was included in the multivariate logistic regression model analysis.

2.10.2 Data Analysis for identification of Drug target Genes mutations

The SeqScape version 2.6 (Applied Biosystems) was used for DNA sequence comparisons and mutations were detected in a respective gene by comparing with the reference *M. tuberculosis* strain H37Rv sequence.

2.10.3 Data Analysis for Genotyping of MTB Isolates

The MIRU-VNTR 24-loci profiles and spoligotyping patterns was used to classify the strains into main phylogenetic lineages using the reference strain collection and identification tools available online at www.Miru vntrplus.org (Allix-Béguec *et al.,* 2008). Identification was carried out as follows: strains was first classified by the simple match approach that is based on the best match with strains of the reference database. The cut of distance for lineage assignment was set to 0.17, and then phylogenetic tree identification was carried out. Besides, for each MIRUVNTR 24-loci pattern a unique MLVA 15–9 code was assigned using the MIRU-VNTR plus nomenclature. For analysis of Spoligotyping, the hybridization patterns were converted into binary and octal formats described by Dale *et al.,* (2001).

Cluster analysis

All results from this study was entered into the Bionumerics program (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium) according to the manufacturers' instructions. Similarities of genotyping patterns among strains was calculated by using the categorical coefficient. A dendrogram was generated by using the unweighted pair group method with arithmetic averages (UPGMA). Minimum spanning tree analysis was done based on MIRU-VNTR typing data by using the categorical coefficient. For the cluster analysis, a cluster was defined as a minimum of two strains harboring identical DNA genotyping patterns (using composite data, MIRU-VNTR 24-loci and spoligotyping) from different patients belonging to the study subjects. Spoligotyping was compared with previously reported strains in the SpolDB4) database (Brudey *et al.,* 2006)

2.10.4 Data Analysis for Evaluation for the performance of GeneXpert MTB/RF Assay

Data were entered using Microsoft Excel and analyzed using SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Descriptive analysis was done to characterize the study population, and diagnostic sensitivity and specificity of GeneXpert MTB/RF Assay were determined using culture results as a reference method with 95% confidence intervals (CI) at α=0·05 Data were entered using Microsoft Excel and analyzed using SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Descriptive analysis was done to characterize the study population, and diagnostic sensitivity and specificity of GeneXpert MTB/RF Assay were determined using culture results as a reference with 95% confidence intervals (CI) at α=0·05 (Agresti and Coull, 1998)

2.11 Data Quality Assurance

To assure data quality, data collectors were trained for two days and the questionnaire was pre-tested before the actual data collection. Completeness, accuracy and consistency of the collected data were checked on daily bases during data collection by supervisors and the principal investigator, where those questionnaires found incomplete, inaccurate and inconsistent were

returned back for data collectors to be filled again. Data were cleaned, edited and coded before data entry and then recoded after analysis.

2.12 Ethical Consideration

The ethical approval and clearance was obtained from Research and Ethical Review Committee of the Addis Ababa University. The study subjects were told about the benefit of being tested. Beside a written and or oral informed consent were taken from each study participant. Permission was also be sought from the hospitals and Health Centers where the study was conducted. Data and specimens was collected and analyzed using codes so that the confidentiality of the patients and test result was maintained throughout the study period.

2.13 Definition

- **New patient:** a patient who has never had treatment for TB, or has taken anti-TB drugs for less than 1 month; the patient may have positive or negative bacteriology.
- **Previously treated patient:** a patient who has received 1 month or more of anti-TB drugs in the past, may have positive or negative bacteriology.
- **Tuberculosis suspect**: any person who presents with symptoms or signs suggestive of TB. The most common symptom of pulmonary TB is a productive cough for more than 2 weeks,1 which may be accompanied by other respiratory symptoms (shortness of breath, chest pains, haemoptysis) and/or constitutional symptoms (weight loss, fever, night sweats, and fatigue).
- **Tuberculosis Case**: a patient with *M. tuberculosis* complex identified from a clinical specimen or one in which a health worker has diagnosed TB and has decided to treat the patient with a full course of TB treatment.
- **Pulmonary tuberculosis:** it refers to a patient with *M. tuberculosis* complex identified from a clinical specimen involving the lung parenchyma. Miliary tuberculosis is classified as

pulmonary TB because there are lesions in the lungs. A patient with both pulmonary and extra-pulmonary TB should be classified as a case of *pulmonary* TB.

- **Extra-pulmonary tuberculosis:** it refers to a patient with *M. tuberculosis* complex identified from a clinical specimen involving organs other than the lungs, e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges.
- **Multi-drug-resiatant- tuberculosis (MDR-TB):** it is defined as *M. tuberculosis that is* resistance to both rifampicin and isoniazid;
- **Extensively drug-resistant (XDR):** is defined as *M. tuberculosis that is* MDR-TB with additional resistance to any fluoroquinolone and at least one of the three second-line injectable drugs: amikacin, capreomycin and kanamycin.
- **MDR-TB suspected case:** a patient who is a case of treatment failure, symptomatic patient who had a close contact with confirmed MDR-TB patient; patient from known high-risk group such as health workers; a patient who remains smear positive after 2 months of treatment (new cases) or remains smear positive after 3 months of retreatment with first-line treatment (retreatment cases such as defaulter, relapse)
- **Relapse:** it is defined as re-emergence of clinical symptoms after stopping anti-**TB** treatment, while this treatment appeared effective initially.
- **Treatment failure:** A patient whose sputum smear or culture is positive at 5 months or later during treatment. Also included in this definition are patients found to harbor a multidrug-resistant (MDR) strain at any point of time during the treatment, whether they are smear-negative or -positive.
- **Defaulter:** A patient whose treatment was interrupted for 2 consecutive months or more.
- **Sensitivity:** it is defined as the number of true positives divided by the total number of true positives and false negatives.
- **Specificity:** it is defined as the number of true negatives divided by the total number of true negatives and false positives.

Chapter 3: Publication I

3. Drug-resistance patterns of Mycobacterium tuberculosis strains and associated risk factors among multi drug-resistant tuberculosis suspected patients from Addis Ababa, Ethiopia

Published at PloS One: PloS One 13 (6) e0197737; doi:10.1371/journal.pone.0197737

3.1 Abstract

Background: Multidrug drug-resistant tuberculosis (MDR-TB) is a major health problem and seriously threatens TB control and prevention efforts globally. Ethiopia is among the 30th highest TB burden countries for MDR-TB with 14% prevalence among previously treated cases. The focus of this study was on determining drug resistance patterns of *M. tuberculosis* among MDR-TB suspected cases and associated risk factors.

Methods: A cross-sectional study was conducted in Addis Ababa from June 2015 to December 2016. Sputum specimens and socio-demographic data were collected from 358 MDR-TB suspected cases. Specimens were analyzed using Ziehl-Neelsen technique, GeneXpert MTB/RIF assay, and culture using Lowenstein-Jensen and Mycobacterial growth indicator tube.

Results: A total of 226 the study participants were culture positive for *M. tuberculosis,* among them, 133 (58.8%) participants were males. Moreover, 162 (71.7%) had been previously treated for tuberculosis, while 128 (56.6%) were TB/HIV co-infected. A majority [122 (54%)] of the isolates were resistant to any first-line anti-TB drugs. Among the resistant isolates, 110 (48.7%) were determined to be resistant to isoniazid, 94 (41.6%) to streptomycin, 89 (39.4%) to rifampicin, 72 (31.9%) to ethambutol, and 70 (30.9%) to pyrazinamide. The prevalence of MDR-TB was 89 (39.4%), of which 52/89 (58.4%) isolates were resistance to all five first-line drugs. Risk factors such as TB/HIV co-infection (AOR=5.59, p=0.00), cigarette smoking (AOR=3.52, p=0.045), alcohol drinking (AOR=5.14, p=0.001) hospital admission (AOR=3.49, p=0.005) and visiting (AOR=3.34, p=0.044) were significantly associated with MDR-TB.

Conclusions: The prevalence of MDR-TB in the study population was of a significantly high-level among previously treated patients and age group of 25-34. TB/HIV co-infection, smoking of a cigarette, alcohol drinking, hospital admission and health facility visiting were identified as risk factors for developing MDR-TB. Therefore, effective strategies should be designed considering the identified risk factors for control of MDR-TB.

Keywords: MDR-TB, TB, Risk factors

3.2 Introduction

Tuberculosis (TB) continues to represent as a global major health challenge to the reduction of morbidity and mortality among millions of people every year. The health of approximately 10.4 million individuals worldwide are impacted annually by TB resulting in approximately 1.8 million TB-related deaths, with the majority (95%) of deaths were reported from resource-limited countries (WHO, 2016). In Sub-Saharan countries, the prevalence of MDR-TB is high especially among previously treated TB cases when contrasted with new cases of TB (Asres *et al.,* 2013). Research has revealed that approximately 500,000 cases of MDR-TB emerge annually every year (Fauci *et al.,* 2008) and that approximately 3% of these cases receive treatment and that more than 100,000 deaths occur annually because of MDR-TB. In addition, as many as 10 % of MDR-TB cases were extensively drug-resistant (XDR) (Falzon *et al.,* 2011). MDR-TB is defined as resistance to both rifampicin and isoniazid; XDR is defined as MDR-TB with additional resistance to any fluoroquinolone and at least one of the three second-line injectable drugs: amikacin, capreomycin, and kanamycin (Parsons *et al.,* 2011).

According to a recent World Health Organization (WHO) report of high TB-related burden countries, Ethiopia was identified as being among the thirty highest TB-burdened nations (TB, TB/HIV and MDR-TB) with TB remaining one of the Ethiopia's leading causes of mortality. According to the 2017 WHO report, the prevalence of MDR-TB in Ethiopia was reported to be 2.7% and 14% among new and previously treated cases respectively with the prevalence of TB/HIV co-infection assessed as being 8% of the affected population (WHO, 2017). Moreover, several studies done in Ethiopia showed that the prevalence of MDR-TB was 31.4% in Jimma (Kedir *et al.,* 2015), 28% in Addis Ababa (Dawit *et al.,* 2008), 46.3% in Addis Ababa (Dereje *et al.,* 2012) and 5% in Northwest Ethiopia (Tessema *et al.,* 2012). Rapid transmission of MDR-TB is a major public health problem globally especially for resource-limited countries and represents a major challenge for TB control program. In addition, a high prevalence of TB, poor treatment, limited access to health care, and several

other related factors have constrained the ability of the sub-Saharan region, including Ethiopia to effectively control MDR-TB (WHO, 2010a). Finally, the rapid transmission of XDR-TB has recently emerged as yet another challenge for TB control program (WHO, 2011a).

Drug-resistant strains of *M. tuberculosis* (MTB) arise from spontaneous chromosomal mutations at a predictable low frequency, but a study done by Gandh *et al.,* revealed that selection pressure that is caused by inappropriate utilize of anti-TB drugs results in the emerging of resistant MTB (Gandhi *et al.,* 2006). Similarly, a study done in Ethiopia identified long treatment, poor treatment follow up and interruption of treatment were identified as risk factors for significant increases in MDR-TB (Selamawit *et al.,* 2013). Other studies done in Ethiopia and China also revealed that HIV infection, cigarette smoking, alcohol drinking, overpopulated, and weak DOTS (Directly Observed Treatment Short-course) program were the major risk factors for spread of MDR-TB infection (Kedir *et al.,* 2015, Dawit *et al.,* 2008, Dereje *et al.,* 2012, Selamawit *et al.,* 2013, Meseret and Demissie, 2014).

The global pattern of MDR-TB is not well known and little information is available regarding MDR-TB strains in a high TB/HIV prevalence countries like Ethiopia. MDR-TB is a result of unsuccessful TB control programs characterized by inappropriate TB treatment, and poor diagnostic capacity. In resource-limited countries such as Ethiopia, MDR-TB is public health threat due to poor adherence to treatment, delay of treatment and shortage of diagnostic center for MDR-TB (WHO, 2010a). In Ethiopia, an MDR-TB suspected case is defined as a patient who is a case of treatment failure; a symptomatic patient who had a close contact with confirmed MDR-TB patient; a patient from known high-risk group such as health workers; a patient who remains smear positive after 2 months of treatment (new cases); or remains smear positive after 3 months of retreatment with first-line treatment (retreatment cases such as defaulter, relapse) (FMOH, 2009). In all such cases, the development of enhanced diagnosis and treatment strategies are essential for controlling transmission of TB especially MDR-TB. Accordingly, this study focused on the identification of drug-

resistance patterns of *M. tuberculosis* strains among MDR-TB suspected patients and the associated risk factors for the development of MDR-TB in the study area.

3.3 Materials and Methods

Study Setting and Design

A cross-sectional institution based survey was conducted between June 2015 and December 2016 in health facilities found in Addis Ababa, the capital city of Ethiopia. The study was conducted in selected health facilities. All specimens were collected from study participants visiting the health facilities in Addis Ababa during the study period. Health facilities which were providing laboratory services for MDR-TB diagnosis were selected from Addis Ababa city. Specimen analysis was performed at Ethiopia Public Health Institute (EPHI) National TB Reference Laboratory, Ethiopia. The sample size was calculated using single population proportion formula considering the assumptions that at 95% confidence level with 5% precision and z value of 1.96 (Barlett *et al.,* 2001), and the 2014 Ethiopian national TB drug resistance survey report showed that the prevalence of drug-resistant TB among previously treated cases was 17.8% (WHO, 2015).

Thus, considering 10% nonresponse rate, the minimum sample size was 248 MDR-TB suspected cases (described in chapter 2 section 2.3). MDR-TB suspected cases are patients who are a case of treatment failure; a symptomatic patients who had a close contact with confirmed MDR-TB patient; patients from known high-risk group such as health workers; patients who remain smear positive after 2 months of treatment (new cases); or remain smear positive after 3 months of retreatment with first-line treatment (FMOH, 2009).

In Ethiopia, drug-resistant TB diagnosis has been carried out using the GeneXpert MTB/RIF assay and phenotypic drug susceptibility testing (DST). However, as GeneXpert MTB/RIF assays and DST are performed in only a few health facilities, MDR-TB suspected cases are referred to GeneXpert MT/RIF diagnostic sites. Since all MDR-TB suspected cases are referred to GeneXpert

MT/RIF diagnostic sites, GeneXpert MT/RIF diagnostic sites found in Addis Ababa City were selected as study sites. Therefore, Addis Ababa Health research and Laboratory services (Addis Ababa Regional referral Laboratory), Teklehiamnot health center, and Saint Peter hospital were the study sites to recruit patients for enrollment in this study. Volunteer MDR-TB suspected patients who visited the health facilities during a study period were included as study participants. MDR-TB suspected patients who were seriously ill or unconscious, patients who were below the age of 18 years old, and patients who were not willing to participate in the study were excluded from the study.

Sputum Specimen collection and Laboratory Analysis

Sputum specimens from patients with PTB were collected into a sterile wide mouth 50 ml falcon tube a volume of 5 to 10 ml, and all specimens were stored at 2-8 ^{o}C at specimen collection sites until transported to EPHI TB laboratory using cold chain. Specimens were analyzed using Ziehl-Neelsen Methods (WHO, 1998) and GeneXpert MTB/RIF assay (Barnard *et al.,* 2012) as the methods described. Moreover, specimens were cultured using LJ (Kent and Kubical, 1985) and MGIT (Siddiqi *et al.,* 2006). methods for better yield; briefly, the specimens were decontaminated with 4% NaOH-NALC and neutralized with phosphate-buffered saline (PBS), and then inoculated on LJ slants at 37 °C for 8 weeks maximum (Kent and Kubical, 1985) and in BACTEC™ MGIT 960 tubes (BD Diagnostics, Sparks, MD, USA) at 37 °C for 42 days maximum (Siddiqi *et al.,* 2006).

All positive culture results were confirmed by using MPT64 antigen detection methods (Capilia TB) (ECDC, 2016). Phenotypic drug susceptibility test for rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) were performed with the Bactec MGIT 960 method. Briefly, 0.5 ml of a bacterial suspension was inoculated into each vial supplemented with reconstitution solution, and a particular drug, such as 1.0 µg/ml for RIF, 0.1 µg/ml for INH, 5.0 µg/ml for EMB 1.0 µg/ml for STR and 100 µg/ml for PZA and 0.1 ml of a bacterial suspension (1:100 dilution) was inoculated into a vial of Growth Control (GC)supplemented with reconstitution solution

only (Siddiqi *et al.,* 2006). *M. tuberculosis* strain H37Rv was used as a sensitive control for the susceptibility testing (details described in Chapter 2, section 2.8.3 and 2.8.4).

Data Analysis

Data were entered using Microsoft Excel and exported to SPSS version 23 for analysis. Data completeness and consistency were checked by running frequencies of each variable. Bivariate analyses were carried out for categorical variables, and odds ratios were used to quantify the strength of association between potential risk factors and MDR-TB. Multiple logistic regressions were used to control the confounding effect of different variables while assessing the effect of each variable on the likelihood of MDR-TB occurrence. A p-value of 0.05 was used as the cut-off point for statistical significance. Variables having a p-value of at most 0.05 in bivariate analysis were included in the multivariate logistic regression model analysis.

Ethical Considerations

Ethical approval was obtained from Research and Ethical Review Committee of the Addis Ababa University and Ethiopian Public Health Institute. Written and or oral informed consent was taken from each study participant, and parent/guardian for those who were under age of 18 years old. Permission was also obtained from study sites. Data and specimens were collected and analyzed using codes so that the confidentiality of the patients and test result were maintained throughout the study period.

3.4 Results

Socio-demographic characteristics of the study participants

A total of 358 MDR-TB suspected cases were enrolled in this study, of which 226 (63.1%) were culture positive for MTB and 5 (1.4%) cases were positive for non-tuberculosis mycobacteria (NTM). Among MTB culture positive cases, majority 133 (58.8%) of cases were males, and 101 (44.7%) of the respondents were in the age group of 24-34 years with an average age of 34.4 years.

Table 3-1: Socio-demographic Characteristics of MDR-TB suspected cases and MDR-TB confirmed cases, Addis Ababa, January, 2017(n=226)

Variable	All MDR-TB suspected cases Number (%) (n=226)	MDR-TB confirmed cases Number (%) (n=89)
Sex		
Male	133 (58.8)	37 (41.6)
Female	93 (41.2)	52 (58.4)
Age Group		
15-24	27 (11.9)	4 (4.5)
25-34	101 (44.7)	53 (59.6)
35-44	68 (30.1)	23 (25.8)
45-54	20 (8.8)	6 (6.7)
Above 54	10 (4.4)	3 (3.4)
Marital Status		
Married	136 (60.2)	54 (60.7)
Single	80 (35.4)	31 (34.8)
Divorced	7 (3)	2 (2.2)
Widow	3 (1.3)	2 (2.2)
Living Region		
AA	208 (92.0)	83 (93.3)
Amhara	1 (0.4)	0 (0)
Dire Dawa	2 (0.9)	2 (2.2)
Oromia	12 (5.4)	4 (4.5)
SNNPR	3 (1.3)	0 (0)
Residence		
Rural	13 (5.8)	4 (4.5)
Urban	213 (94.2)	85 (95.5)
Education status		
College	29 (12.8)	15 (16.9)
High School	72 (31.8)	26 (29.2)
Elementary	57 (25.1)	18 (20.2)
RandW	54 (23.9)	22 (24.3)
Illiterate	14 (6.2)	8 (9.0)
Religion		
Muslim	46 (20.4)	19 (21.3)
Orthodox	152 (67.3)	61 (68.5)
Protestant	28 (12.4)	9 (10.1)
Monthly Income in ETB		
No Income	3 (1.3)	6 (6.7)
100-1000	47 (20.8)	15 (16.9)
1001-2000	101 (44.7)	32 (36.0)
2001-3000	42 (18.6)	23 (25.8)
3001-4000	26 (11.5)	13 (14.6)
4001-5000	7 (3.1)	0 (0)
Occupation	3 (1.3)	6 (6.7)
Daily Laborer	28 (12.4)	9 (10.1)
Government Worker	30 (13.3)	14 (15.7)
House wife	26 (11.5)	13 (14.6)
Private Worker	72 (31.9)	23 (25.8)
Self-employed	67 (29.6)	29 (32.6)
Unemployed	3 (1.3)	1 (1.1)
Number of rooms in residence		
1-2	173 (76.5)	64 (71.9)
3-4	52 (23)	24(27)
5-6	1 (0.5)	1 (1.1)

ETB: Ethiopian Birr, SNNPR: Southern Nations, Nationalities, and Peoples' Region

Majority 213 (94.2%) of the respondents were living in an urban environment. Married individuals accounted for the majority 162 (60.2%) the cases but 80 (35.4%) were never married at all. Most 152 (67.2%) of the respondents were Orthodox by religion and majority 134 (53.1%) of the cases were from Amhara and Oromo ethnic group. Seventy two (31.8%) of the respondents attended high schools, and they were employed at a private organization and 96 (42.5%) of participants had less than 1000 Birr (50 USD) income per month. More than half, 117 (51.7%) of the participants had 2 rooms in their residence. A majority [174 (77%)] of the participants lived in families with more than two family members (Table 3.1, Figure 3.1).

TB and treatment related conditions among MDR-TB suspected case

Among the MDR-TB suspected cases, 196 (86.7%) cases were AFB positive and 128 (56.6%) cases were TB/HIV co-infected. One hundred sixty two (71.7%) cases were previously treated cases that had a history of TB treatment for more than a month in addition to this, 77 (34.1%) cases had a history of family member infected by TB. Among the previously treated cases (n=162), 148 (91.4%) were relapse cases and the remaining 9 (5.6%), and 5 (3.1%) were treatment failure and defaulter cases, respectively.

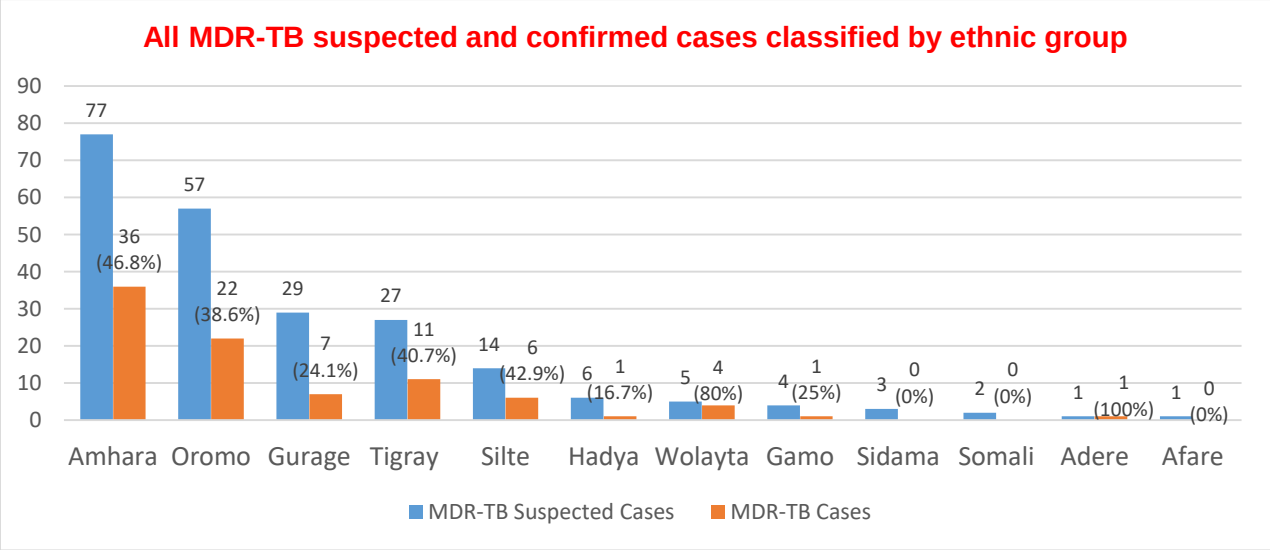


Figure 3-1: The proportion all MDR-TB Suspected and confirmed cases classified by ethnic group (12 ethnic groups)

In addition, 26 (16.0%) cases had discontinued anti-TB drug during treatment time, of these 16 (61.5%) cases had discontinued anti-TB drugs for a month or more and most 24 (92.3%) of the patients discontinued one time during their treatment period. One hundred and ninety (84.1%) cases were visiting health facilities for other reasons and 51 (26.8%) cases were admitted to hospital. Eight-five (37.6%) cases had an antibiotic treatment history for other diseases, of which 32 (37.7%) had interrupted antibiotic treatment for more than once. Moreover, 42 (18.6%) participants were self-reported frequent consumers of alcohol and 27 (11.9%) reported themselves as being frequent cigarette smokers (Table 3.2).

M. tuberculosis identification and drug susceptibility test results

Among 226 *M. tuberculosis* isolates, the majority 123 (54.4%) of isolates were resistant to at least one of the five first-line anti TB drugs (RIF, INH, PZA, EMB, and STR), however, the remaining 104 (46.0%) isolates were susceptible to all first-line anti TB drugs. Moreover, resistance to INH, STR, RIF, EMB and PZA were 110 (48.7%), 94 (41.6%), 89 (39.4%), 72 (31.9%), and 70 (30.9%), respectively. Furthermore, the proportion of drug-resistance pattern among previously treated cases (n=162) were 87 (53.7%), 75 (46.3%), 73 (45.1%), 59 (36.4%), and 59 (36.4%), and 57 (35.2%), INH, STR, RIF, EMB and PZA, respectively (Table 3.3). However, 3 (1.3%) isolates had discordant results from MGIT and GeneXpert MTB/RIF methods for DST, of which 2 isolates had RIF susceptible results from MGIT but they were found to be RIF resistant by GeneXpert MTB/RIF assay. The other one isolate had a resistant result from MGIT with GeneXpert MTB/RIF susceptible result. Moreover, when we evaluated the performance of GeneXpert MTB/RIF method against MGIT for DST, we found that sensitivity result was 99.1% with 99.6% specificity.

Patterns of multidrug-resistance

The prevalence of multi-drug-resistant TB was found to be 89 (39.4%), of which 73 (82.0 %), 65 (73.0%) and 63 (70.8%) were additionally resistant to STR, EMB and PZA anti-TB drugs, respectively.

Table 3-2: TB disease and other related conditions among MDR-TB suspected and MDR-TB confirmed cases, Addis Ababa, January, 2017(n=226)

Variable	All MDR-TB suspected cases Number (%)	MDR-TB confirmed cases Number (%)
Family member Previously TB infected		
No	149 (65.9)	66 (74.2)
Yes	77 (34.1)	23 (25.8)
Treatment history of TB infected families		
No	3 (3.9)	0 (0)
Yes	74 (96.1)	23 (100)
Previously TB Infected		
No	64 (28)	16 (18)
Yes	162 (71.7)	73 (82)
Treatment history of previously TB infected cases		
No	0 (0)	0 (0)
Yes	162 (100)	73 (100)
Treatment interruption previously TB treated cases		
No	136 (84.0)	63 (86.3)
Yes	26 (16.0)	10 (13.7)
Duration of treatment interruption in days		
7-21	7 (26.8)	2 (20)
30-45	16 (61.5)	7 (70)
150	1 (3.8)	0 (0)
Unknown	2 (7.7)	1 (10)
Frequency of drug interruption		
1	24 (92.2)	9 (90)
3	1 (3.9)	0 (0)
Unknown	1 (3.9)	1 (10)
DOT treatment		
No	1 (0.6)	1(1.4)
Yes	161 (99.4)	72(98.6)
TB treatment history		
New	64 (28.3)	16 (18)
Retreatment	162 (71.7)	73 (82)
TB history		
New	64 (28.3)	16 (18)
Defaulter	5 (2.2)	3 (3.4)
Relapse	148 (65.5)	63 (70.8)
Treatment failure	9 (4.0)	7 (7.9)
AFB Results		
Negative	30(13.3%)	2(2.2%)
Positive	196(86.7%)	87(97.8%)
HIV status		
Positive	128 (56.6)	71 (79.8)
Negative	98 (43.4)	18 (20.2)
Antibiotic treatment history (frequently)		
No	141 (62.4)	45 (50.6)
Yes	85 (37.6)	44 (49.4)
Antibiotic treatment interruption		
No	53 (62.3)	29 (65.9)
Yes	32 (37.7)	15 (34.1)
Alcohol drinking frequently		
No	184 (81.4)	61 (68.5)
Yes	42 (18.6)	28 (31.5)
Cigarettes smoking		
No	199 (88.1)	73 (82)
Yes	27 (11.9)	16 (18)

Health facility visiting		
Yes	190 (74.1)	82(92.1)
No	36 (15.9)	7 (7.9)
Hospital admission		
No	175 (77.4)	55 (61.8)
Yes	51 (22.6)	34 (38.2)

AFB: Acid fast Bacilli; DOT: Directly Observed Treatment; HIV: Human Immunodeficiency Virus

In addition, among multi-drug-resistant TB cases, 52/89 (58.4%) were resistance for all first-line anti TB drugs (RIF, INH, PZA, EMB, and STR). Moreover, the prevalence of Multi-drug-resistant TB among previously treated cases was 73/162 (45.1%) whereas 16/64 (25%) were among new cases. A higher multi-drug-resistance rate was observed among previously treated cases 73/89 (82.0%) compared with new cases, and a substantial drug-resistance pattern was observed on STR, PZA, and EMB drugs with 64 (87.7%), 53 (72.6%) and 50 (68.5%) isolates respectively. Out of 89 MDR-TB confirmed cases, more than three-fourth, [71 (79.8%)] were HIV positive patients, and majority 87 (97.8%) of the MDR-TB cases were AFB positive (Table 3.3).

Seventy-three (82.0%) MDR-TB cases were previously treated cases and the remaining 16 (18.0%) were new cases. Moreover 23 (25.8%) MDR-TB cases had a history of family member infected by TB. Among the previously treated cases, the majority [63 (86.3%)] of the MDR-TB cases were relapse cases, and 7 (9.6%) cases had discontinued their use of an anti-TB drug during treatment and 5/7 (71.4%) the cases discontinued anti-TB drugs for more than a month. Moreover 82 (92.1%) MDR-TB cases were visiting health facilities for other reasons and 20 (22.5%) cases were admitted to hospital. More than a quarter, [23 (25.8%)] had an antibiotic treatment history for other diseases, while 8/23 (34.7%) of these cases had interrupted antibiotic treatment more than once. The majority of MDR-TB cases 52 (58.4%) were males and 85 (95.5%) MDR-TB cases were lived in an urban environment. Fifty-three (59.6%) of the MDR-TB cases were in the age group of 25-34 years and 54 (60.7%) the cases were married and more than half, [58 (65.2%)] of the cases were from Amhara and Oromo ethnic group (Table 3.2, Figure 3.1).

Table 3-3: Drug resistance Pattern in *M. tuberculosis* complex isolates among retreatment and new MDR-TB suspected cases Addis Ababa, January, 2017(n=226)

Drug Resistance Pattern	All case (n=226) Number (%)	Previously treated cases (n=162) Number (%)	New cases (n=64) Number (%)
Any Resistance	**123 (54.4)**	**95 (58.6)**	**28 (43.8)**
EMB	72 (31.9),	59 (36.4)	13 (20.3)
INH	110 (48.7),	87 (53.7)	23 (35.9)
STR	94 (41.6)	75 (46.3)	19 (29.7)
PZA	70 (30.9)	57 (35.2)	13 (20.3)
RIF	89 (39.4)	73 (82)	16 (25.0)
Mono Resistance	**20 (8.8)**	**12 (7.4)**	**8 (12.5)**
EMB	4 (1.8)	4 (2.5)	0 (0.0)
INH	7 (3.1)	4 (2.5)	3 (4.7)
STR	7 (3.1)	3 (1.9)	4 (6.3)
PZA	2 (0.9)	1 (0.6)	1 (1.6)
Multi drug Resistance(MDR)	**89 (39.4)**	**73 (45.1)**	**16 (25.0)**
RIF + INH	5 (2.2)	5 (3.1)	0 (0)
RIF + INH + EMB	3 (1.3)	1 (0.6)	2 (3.1)
RIF + INH + STR	10 (4.4)	8 (4.9)	2 (3.1)
RIF + INH + PZA	2 (0.9)	1 (0.6)	1 (1.6)
RIF + INH + EMB + STR	6 (2.7)	6 (3.7)	0 (0)
RIF + INH + EMB + PZA	4 (1.8)	3 (1.9)	1 (1.6)
RIF + INH + STR + PZA	7 (3.1)	6 (3.7)	1 (1.6)
RIF + INH + EMB + STR + PZA	52 (23.0)	43 (26.5)	9 (14.1)
Poly Resistance* (Non MDR)	**13 (5.8)**	**9 (5.6)**	**4 (6.3)**
EMB + INH	2 (0.9)	1 (0.6)	1 (1.6)
INH + STR	6 (2.7)	3 (1.9)	3 (4.7)
EMB + INH + STR	1 (0.4)	1 (0.6)	0 (0.0)
INH + STR + PZA	3 (1.3)	3 (1.9)	0 (0.0)
EMB + INH + STR + PZA	1 (0.4)	1 (0.6)	0 (0.0)

INH: Isoniazid, RIF: Rifampicin, STR: Streptomycin, EMB: Ethambutol, PZA: Pyrazinamide
*Poly Resistance is a drug resistance to two and more drugs without the combination of INH and RIF

Risk factors associated with multidrug resistance development

In a univariate analysis of different variables with the development of MDR-TB, TB/HIV co-infection, previously TB infected cases, a family member who had previously TB infected cases, antibiotic taking, alcohol drinking, age group between 25 and 34 years, cigarette smoking, health facility visits and hospital admission appear to represent significant risk factors for MDR-TB($p<0.05$). Moreover, multivariate analysis indicated that MDR-TB is significantly associated with hospital admission (AOR= 3.49, p=0.005), health facility visit, (AOR= 3.34, p=0.044), TB/HIV co-infection (AOR= 5.59, p=0.00), alcohol drinking (AOR= 5.14, p=0.001) and cigarette smoking (AOR= 3.52, p=0.045).

Table 3-4: Univariate and multivariate Logistic regression result of risk factors for development of MDR-TB, Addis Ababa, January, 2017(n=226)

Variable	MDR-TB Number (%)		Crude odds ratio (95% CI)	P-Value	Adjusted Odd Ratio (95% CI)	P-Value
	Yes	No				
Age group						0.261
15-24	4 (14.8)	23 (85.2)	1		1	
25-34	53 (52.5)	48 (47.5)	6.35 (2.05 19.70)	0.001	3.73 (0.51 27.37)	
35-44	23 (33.8)	45 (66.2)	2.94 (0.91 9.51)		0.65 (0.13 3.27) 0.78	
45-54	6 (30.0)	14 (70.0)	2.46 (0.59 10.29)		(0.34 9.28)	
55 ≥	3 (30.0)	7 (70.0)	2.46 (0.44 13.75)		2.29 (0.34 15.36)	
Cigarettes smoking						0.045
Yes	73 (36.7)	126 (63.3)	2.51 (1.11 5.70)	0.045	3.523 (1.03-12.05)*	
No	16 (59.3)	11 (40.7)	1		1	
Alcohol drinking						0.001
Yes	61 (33.2)	123 (66.8)	4.03 (1.98 8.12)	0.000	5.142 (1.98 13.33)*	
No	28 (66.7)	14 (33.3)	1		1	
HIV status						0.000
Positive	71 (55.5)	57 (44.5)	5.54 (2.98 10.28)	0.000	5.59 (2.65 11.75)*	
Negative	18 (18.4)	80 (81.6)	1		1	
Antibiotic treatment history						0.106
Yes	45 (31.9)	96 (68.1)	2.29 (1.32 3.98)	0.000	1.829 (0.88 3.80)	
No	44 (51.8)	41 (48.2)	1		1	
TB treatment history						
Retreatment	73 (45.1)	89 (54.9)	2.46 (1.91 4.69)	0.000	2.21(0.8 5.89)	0.110
New	16 (25)	48 (75)	1		1	
TB history						
New	16 (25.0)	48 (75.0)	1	0.012	1	0.067
Defaulter	3 (60.0)	2 (40.0)	4.50 (0.69 29.38)		8.26 (0.86 78.95)	
Relapse	63 (42.6)	85 (57.4)	2.22 (1.16 4.27)		3.56 (0.26 50.16)	
Treatment failure	7 (77.8)	2(22.2)	10.5 (1.97 55.8)		2.78 (0.48 16.145	
Previously TB infected Family member				0.037		0.541
Yes	66 (44.3)	83 (55.7)	0.54 (0.30 0.96)		0.67 (0.18, 2.45)	
No	23 (29.9)	54 (70.1)	1		1	
Health facility visit						0.044
Yes	7 (19.4)	29 (80.6)	3.15 (1.31 7.54)	0.019	3.336 (1.03 10.78)*	
No	82 (43.2)	108 (56.8)	1		1	
Hospital admitted				0.000		0.005
No	55 (31.4)	120 (68.6)	4.36 (2.25 8.48)		3.492 (1.45 8.40)*	
Yes	34 (66.7)	17 (33.3)	1		1	

***** The odds ratio *indicated that there was significant Association between dependent variable (*MDR-TB*) and independent Variables*

Patients admitted to hospital and visited health facilities and those who were self-reported as being frequent cigarette smokers were three times more likely to develop MDR-TB when compared to those who did not fit these profiles. Moreover, TB/HIV co-infection and drinking alcohol were five times more likely to have MDR-TB when compared with those HIV negative cases and TB patients who did not drink alcohol (Table 3.4).

3.5 Discussion

In this study, about 72% of cases were previously treated cases that had a history of TB treatment for more than a month and 34.1% cases had a history of a family member infected by TB. In addition, 16.0% of the cases had discontinued anti-TB drug during the treatment period. More than 56% of the cases were TB/HIV co-infected patients, and about 80% of the TB/MDR-TB cases were HIV positive patients. The high prevalence in our study could be attributed to the fact that HIV-positive patients are more likely to develop TB/MDR-TB than HIV negative patients due to their immunocompromised status. In supporting our finding, several studies revealed that HIV infection was the major associated risk factor for the spread of MDR-TB infection in population (Kedir *et al.,* 2015, Dawit *et al.,* 2008, Dereje *et al.,* 2012, Selamawit *et al.,* 2013, Meseret and Demissie, 2014). Moreover, the study also found that more than 95 % of the MDR-TB cases lived in an urban environment. Evidence from a previous study showed that patients who live in an urban area are more likely to develop drug-resistant TB due to slums/overcrowded area that favor for transmission of TB/MDR-TB (Kedir *et al.,* 2015).

Furthermore, the highest rate of MDR-TB patients was also in the age group of 25–34 years, a finding that is consistent with other studies done in Ethiopia (Kedir *et al.,* 2015, Amare *et al.,* 2011, Yohanes *et al.,* 2012). The highest rate of MDR-TB in this age group might possibly be due to this age cohort's high mobility and high-risk behavior could possibly expose them to greater risk of acquiring TB as well as to a tendency to interrupt TB treatment. In another finding, this study identified that 54% of the study's population was resistant to any first-line anti-TB drug, a finding that was lower than previous studies done in Addis Ababa (72.9%) (Dereje *et al.,* 2012) and Southwest, Ethiopia (58.6%) (Kedir *et al.,* 2015). In accounting for this difference, it is possible that, as our study included both previously treated and new cases, a new TB patient is less likely to develop drug-resistant TB.

The highest proportion of drug resistance was observed for INH (49%). This is comparable to the studies done in Southwest Ethiopia (51%) (Kedir *et al.,* 2015) and Addis Ababa (56.1%) (Dereje *et al.,* 2012). However, as our finding on 49% INH resistance was slightly higher than previous studies done in Ethiopia such as 44% (Dawit *et al.,* 2008) and 42.7% (Dereje *et al.,* 2012), the high proportion of isoniazid resistance has significant implications since it is an essential drug during the course of TB treatment and a prophylaxis for latent TB infected individuals, HIV/AIDS patients and household contacts of smear-positive pulmonary TB cases. In addition, our study found that, at a rate of 41.6%, streptomycin resistance was comparable with a study done in Southwest, Ethiopia (42.9%) (Kedir *et al.,* 2015), although appreciably higher than other Ethiopian studies such as 21% (Dereje *et al.,* 2012), and 28 % (Dawit *et al.,* 2008). The high resistance to streptomycin could be due to the common use of the drug for treatment of any bacterial infections, poor treatment practice and early introduction for treatment (Gillespie 2002).

The proportion of drug resistance for rifampicin drug was rated third with 39.4 % and all rifampicin drug-resistant cases were also resistant to Isoniazid which are MDR-TB cases. This finding concurs the present practice of TB programs to use RIF resistance as a surrogate marker for MDR-TB diagnosis and second-line anti-TB drugs treatment initiation. Our finding was higher than the findings of previous studies done in Addis Ababa (33.3%) (Dawit *et al.,* 2008) and Southwest, Ethiopia (32.9%) (Kedir *et al.,* 2015). The high prevalence of our finding might be that the study was conducted on the population of MDR-TB suspected patients. Furthermore, the ethambutol drug resistance was ranked fourth with 31.9 %. It is comparable to a study done in Southwest, Ethiopia that the proportion of any ethambutol resistance was about 29% (Kedir *et al.,* 2015). Although it is the first-line drug, it is also included in the regimen of second-line drugs for MDR-TB treatment. Hence the high rate of ethambutol resistance could be a challenge for MDR-TB treatment in the future (Hoek *et al.,* 2009). Furthermore, resistance to PZA was 30.9%, it is well known that PZA is a cornerstone anti TB drug because of its unique ability to eradicate persistent bacilli, that allowed

treatment shortening from 9 months to 6 months (Mitchison 1985), and it is continuing as an important drug for susceptible and MDR-TB treatment (Shi *et al.,* 2014).

In terms of prevalence of MDR-TB, the prevalence of MDR-TB was 39.4%, our finding was somewhat higher than the previous study done in Southwest, Ethiopia 31.4% (Kedir *et al.,* 2015), and compared with Ethiopian national prevalence for previously treated cases, it was twofold higher (17.8%) (WHO, 2017). In addition, the proportion of MDR-TB among previously treated was 45.1%. This finding is in agreement with a study done in Addis Ababa, Ethiopia (46.3%) (Dereje *et al.,* 2012) and India (47.1%) (Hanif *et al.,* 2009). However, it was higher than other studies conducted in Ethiopia (28%) (Dawit *et al.,* 2008) and Northwest Ethiopia (13.9%). The plausible reasons for high prevalence in our finding might be that the study was conducted among the population of MDR-TB suspected patients; , the nature of this population included in the studies, and there might also be geographical variation in the level of drug resistance. Our assumption was supported by Feleke and colleagues (Feleke *et al.,* 2015).

In another finding, the percentage of MDR-TB among previously treated cases was significantly higher (45.1%) compared to new TB cases (25%). It is well documented that previously treated cases are more likely to develop MDR–TB than new patients (Kelemework *et al.,* 2015). High rates of MDR-TB among previously treated cases can be influenced by the acquisition of resistance in the intensive and continuation phases of treatment or by the rate of primary MDR-TB infection (Sharma *et al.,* 2011). In addition, the rate of MDR-TB among new cases was 25% was higher than Ethiopian national prevalence for new cases was 2.7% (WHO, 2017), and in other studies done in Ethiopia including those in Debre Markos (10.7%) (Wondemagegn *et al.,* 2017), Northwest Ethiopia (2.3%) (Feleke *et al.,* 2015), and East Gojjam (1.29%) (Kelemework *et al.,* 2015). This finding might indicate a significant public health threat given that there would appear to be a progressive MDR-TB transmission in the population. So this finding could be a good indicator of a need to strengthen

the health system towards a more effective TB treatment, diagnostic, and prevention and control program.

Furthermore, while it is well known that the drug-resistant TB is a result of chromosomal alterations due to mutations or deletions, there are several factors related to TB control program that have a significant impact on the increasing and transmission of drug-resistant TB (Migliori *et al.,* 2011). Our study revealed that MDR-TB infection had a statistically significant association with patients admitted to hospital ($p<0.005$), patients who visited health facilities ($p<0.005$), HIV positive patients ($p<0.005$), patients who were frequent cigarette smokers ($p<0.005$) and patients who frequently drink alcohol ($p<0.005$). All of these factors would appear to be predictors for MDR-TB. This finding is in agreement with studies done in Addis Ababa, Ethiopia (Dereje *et al.,* 2012, Dawit *et al.,* 2008), and China (Liang *et al.,* 2012). Moreover, several pieces of evidence revealed that HIV/AIDS, overcrowding and lack of compliance with DOTS program, are also the potential risk factors for the development of MDR-TB infection (Kedir *et al.,* 2015, Selamawit *et al.,* 2013, Meseret and Demissie, 2014, Barroso *et al.,* 2003).

In another part of this study, we tried to evaluate the performance of GeneXpert MTB/RIF method against MGIT, which is a standard method, for DST, we found that 3 isolates had discordant results from MGIT and GeneXpert MTB/RIF methods (99.1% sensitivity and 99.6% specificity), two isolates had RIF susceptible results from MGIT with GeneXpert MTB/RIF resistance results. This situation might be due to the existence of *M. tuberculosis* strains with borderline of susceptible (Van and Victor, 2005). Moreover, one isolate was found to be resistance from MGIT and susceptible to GeneXpert MTB/RIF assay, It is well documented that about 5% RIF resistance isolates did not have any mutation in the rpoB gene, and the mechanism of resistance could be due to intrinsic drug resistance mechanism in which it is attributed to its unique cell wall properties, including the presence of mycolic acids, which constitute a very hydrophobic barrier responsible for resistance

to certain antibiotics (Karakousis *et al.,* 2004). So this might be preliminary finding for further study on the performance of GeneXpert MTB/RIF assay among MDR-TB suspected cases in Ethiopia.

In general, the findings presented in this paper would tend to confirm that patient progressive acquisition of drug resistance during TB treatment is a significant contributor to higher rates of MDR-TB since anti-TB drug treatment suppresses the growth of susceptible TB isolates while, at the same time, favor the multiplication of the existing drug-resistant isolates as described by Feleke *et al.,* (Feleke *et al.,* 2015). MDR-TB control programs currently focus on factors implementing the guidelines for TB control programs such as early case detection, treatment adherence, infection prevention and administrative and logistic issues (Kariuki, 2010). As this study provides information on patterns of drug-resistant TB and associated risk factors among previously treated and new cases, it is proposed that this study's findings could be applied to an increased understanding of factors associated with the development of MDR-TB in the population and, hence, to ways in which to improve planning associated with ways by which to reduce MDR-TB.

3.6 Conclusion

In conclusion, the present study has revealed that the prevalence of multidrug-resistant tuberculosis in the study area was higher compared to WHO data and previous studies done in Ethiopia and that the proportion of MDR-TB among previously treated patients and young age group was also higher than previous studies. The major risk factors for the development of MDR-TB were TB/HIV co-infection, frequent cigarette smoking, frequent consumption of alcohol, hospital admission, and a history of visits to health facilities. Finally, this study would conclude that, as a major public health threat is represented by the finding that there is a progressive MDR-TB transmission in the population especially in the productive age group of the population, actions should be taken to improve outreach to populations at risk of MDR-TB if Ethiopia is to avoid an environment in which MDR-TB continues to increase its impact on the health of the nation.

Therefore, it is recommended that TB patients suspected of MDR-TB should be identified in a timely manner and treated according to treatment guideline, and the country should focus its efforts on designing and developing a strategy towards early detection and treatment of MDR-TB cases in the population, and monitor systems to investigate the trend of MDR-TB incidence and efficacy of MDR-TB treatment regimens. Moreover, further studies should be supported to determine the transmission dynamics of multidrug-resistant strains using genotyping tools as well as studies devoted to increasing and refining the public health community's understanding of risk factors for the development of MDR-TB in the population.

Chapter 4: Publication II

4. Analysis of Gene Mutations Associated with Anti-TB Drugs Resistance among Mycobacterium tuberculosis strains isolated from MDR-TB suspected patients in Addis Ababa, Ethiopia

Will be submitted to the International Journal of Tuberculosis and Lung Disease (IJTLD)

4.1 Abstract

Background: Drug-resistant tuberculosis (TB) is a major health problem and threatens to TB control program. It is a result of unsuccessful TB control programs, and associated with mutations in drug target genes. Understanding of the pattern of mutations is important for TB control. Thus, the aim of this study was to identify mutation associated with first-line anti-TB drug target genes.

Methods: A total of 209 isolates were included in the study. Phenotypic drug susceptibility test was done for rifampicin, isoniazid, pyrazinamide, ethambutol, and streptomycin drugs. Sequencing was also performed for mutation analysis in rpoB, katG, fabG1-inhA, embB, pncA, rrs and rpsL drug target genes.

Result: Based on the DST result, the proportions of resistance to isoniazid, streptomycin, rifampicin, ethambutol, and pyrazinamide were 110 (48.7%), 94 (41.6%) 89 (39.4%) 72 (31.9%), and 70 (30.9%) respectively. Ninety (43.1%) isolates had the mutations in rpoB gene, of these 84 (95.5%) isolates were rifampicin resistance, and the highest [61 (67.8%)] nucleotide substitution occurred at codon S531L/W, and other mutations were found at codons Q513P, D516V, H526Y/S/D/L, L533P, L538P/V and R529P. Mutations in katG gene were observed 101 (48.3%) isolates, and 98 (98.0%) had at codon S315T, besides 7 (3.3%) isolates had a mutation in fabG1-inhA promoter region. Moreover, 68 (30.1%) isolates had a mutation in pncA gene with 25 different mutations. Another mutation was found in an embB gene with 73 (34.9%) isolates, and majority 48 (65.8%) of the mutations were found at codon 306 (M306I/V/L). Furthermore, mutations in rrs and rpsl genes found in 56 (26.8%) isolates with high 50 (89.3%) proportion in rpsL gene at codon K88R/T and K43R, however, no mutation was found in 36 (38.7%) STR resistant isolates.

Conclusion: The highest proportions of mutations were observed at codon 531, 315, 306, 65, and 88 in rpoB, katG, embB, pncA, and rpsl genes respectively. The identified mutations in circulating strains were similar to the mutations reported globally, but there were discrepancy results between phenotypic and molecular analysis, suggesting further study.

Keywords: *M. tuberculosis*, MDR-TB, mutation, rpoB, katG, embB, pncA, rpsL,

4.2 Introduction

Tuberculosis (TB) continues as major health problem globally. It causes a disease among millions of people each year and it is the second leading cause of death from an infectious disease worldwide. In 2016 there were about 10.4 million TB cases, and more than 1.3 million TB deaths and about 85% of TB deaths occurred in African and South-East Asia Region. Moreover, drug-resistant (DR) TB is one of the major global threat, and according to WHO report, in 2016, there were about half a million multidrug-resistant TB (MDR-TB) patients, MDR-TB is defined as resistance to both isoniazid and rifampicin, the two most effective first-line drugs, and the global prevalence of MDR-TB was 4.1% and 19% among new and previously treated cases respectively. Almost half (47%) of these cases were from India, China and the Russian (WHO, 2017). In addition to this, another report showed that the prevalence of MDR-TB is also high in Sub-Saharan countries especially among previously treated TB cases (Asres *et al.,* 2013).

Ethiopia is one of the 30 high burden countries for TB, TB/HIV and MDR-TB globally, and a disease of tuberculosis remains one of the leading causes of mortality in the country. According to 2017 WHO report, the prevalence of MDR-TB was 14% among previously treated and 2.7% among new cases. In addition to this, 8% of TB cases were TB/HIV co-infected patients (WHO, 2017). However, different studies conducted in the country shows variation in the prevalence of MDR-TB from regions to regions, for instance 31.4% in Jima (Kedir *et al.,* 2015), 46.3% in Addis Ababa (Dereje et. al., 2012) and 5% in Northwest Ethiopia (Tessema et. al., 2012). Indicating that there is a high need of generating information concerning the prevalence of mutations associated with anti-TB drugs in the local settings. The emergence of MDR-TB is a major public health threat for the populations of resource-limited setting and become a significant obstacle for TB control programs. In addition to this, a high prevalence of infectious diseases and limited access to health care facilities making worst the consequence of MDR-TB. Moreover, poor treatment outcomes,

longer treatment time, treatment costs, and many more complications make MDR-TB a complex disease in the sub-Saharan region (WHO, 2010b).

The high prevalence and transmission of MDR and extensively drug-resistant (XDR) strains of *M. tuberculosis* (MTB) poses the challenges for effective therapeutic options and infection control (WHO, 2011b). XDR is defined as MDR TB with additional resistance to any fluoroquinolone (FQ) and at least one of the three second-line injectable drugs: kanamycin (KAN), amikacin (AMK), and capreomycin (CAP) (Parsons *et al.,* 2011). Drug-resistant strains of *MTB* arise from spontaneous chromosomal mutations at a predictable low frequency but study revealed that selection pressure that is caused by misuse of anti-TBdrugs, such as using mono-therapy, results in an increase in the frequency of resistant mutants in population (Gandhi *et al.,* 2006). In high burden countries, several TB patients have been living with drug-resistant TB due to the lack of appropriate diagnosis and treatment system, and they can easily transmit it to the population. Most of the drug-resistant TB is associated with the mutation in genes or promoters of genes activating the drug or encoding the drug targets and associated with mutations in several genes (Ong *et al.,* 2010).

Studies showed that rifampicin resistance is mediated by mutations in an 81 bp region of the *rpoB* gene and 96% of *MTB* strains are due to mutations in an 81 base-pair region of the *rpoB* gene encoding the beta subunit of RNA polymerase. Mutations associated with INH resistance occur mainly in the katG gene at codon 315 that encodes a catalase-peroxidase enzyme, the inhA gene and regulatory region, and the ahpC regulatory region (Piatek, 2000) and mutation atkatG gene occurs in 50–80% of strains (Zhang and Yew, 2009), and 10% of the strains have a mutation in the *inhA* locus (Ramaswamy and Musser, 1998). A mutation that associated with pyrazinamide (PZA) and Ethambutol (EMB) resistance occurs in a pncA gene encoding pyrazinamidase/ nicotinamidase (Scorpio and Zhang, 1996) and *embB* operon respectively. Studies showed that more than 72% of PZA and 68% of EMB resistant strains had a mutation at *pncA* genes

(Sreevatsan *et al.,* 1997a) *and embB* genes (Sreevatsan *et al.,* 1997b). Moreover, Streptomycin (STR) resistance-associated mutations occurrs in rpsL gene that encode the S12 protein and rrs gene encodes the 16S rRNA, and accounting for about 50% and 20% of STR-resistant strains respectively (Telenti *et al.,* 1997).

However some genes responsible for drug resistance are not well known, and a little information is available on the pattern of gene mutation associated with anti-TB drugs in sub-Sahara countries. Especially in Ethiopia, there is no information on Single Nucleotide Polymorphisms (SNPs) data on drug target gene associated with drug resistance. So analyzing mutations associated with drug target genes would help to find the extent of drug-resistant TB and the pattern of SNPs in the drug target genes that might be used as molecular markers for rapid diagnosis of MRD-TB. There is also a concern that these strains could spread around the world, stressing the need for additional control measures, such as new diagnostic methods, better drugs for treatment, and a more effective vaccine. Therefore, the aim of this study was to identify mutations of target genes (rpoB, katG, fabG1(mabA)-inhA, embB, pncA, rrs, and rpsL) related to resistance to RIF, INH, EMB, PZA and STR drugs among MDR-TB suspected cases in the high incidence setting from Addis Ababa, Ethiopian.

4.3 Materials and Methods

Study Setting and Design

A cross-sectional survey was conducted between June 2015 and December 2016 in selected health facilities found in Addis Ababa, Ethiopia. Data and sputum specimens were collected from study participants visiting the health facilities during the study period. Sample size was calculated using single population proportion formula considering the assumptions that at 95% confidence level with 5% precision and z value of 1.96 (Barlett *et al.,* 2001) and 17.8% prevalence of MDR-TB among previously treated cases.(WHO, 2015) Thus considering 10% nonresponse rate, the final sample size calculated was 248 (described in chapter 2, section 2.3). Since Addis Ababa

Health research and Laboratory services, Saint Peter hospital and Teklehiamnot health center were a referral diagnostic sites for MDR-TB suspected patient in Addis Ababa, they were selected as study sites. Volunteer an MDR-TB suspected patients who visited the health facilities during the study period were included as study participants. MDR suspected TB patients who were seriously ill or unconscious, below the age of 18 years old and unwilling to participate in the study were excluded from the study.

Sputum Specimens collection and storage

Sputum specimens from MDR-TB suspected patients were collected from the selected health facilities for the study. A volume of 5 to 10 ml sputum specimen produced by a deep cough from the patient was collected into a sterile wide mouth 50 ml falcon tube. All specimens were stored at 2-8^{o}C at collection sites until transported to Ethiopian Public Health Institute National TB Reference laboratory using cold chain.

Laboratory Analysis and Drug Susceptibility Test (DST)

Sputum specimens were analyzed using Ziehl-Neelsen Methods (WHO, 1998), GeneXpert MTB/RIF assay (Barnard *et al.,* (2012), Culture (LJ and MGIT) methods as described previously by Kent and Kubical (1985) and Siddiqi and Rüsch (2006). All positive cultures were further confirmed using MPT64 antigen detection methods (Capilia TB) (ECDC, 2016). Phenotypic drug susceptibility test for rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) were performed with the Bactec MGIT 960 method as described by Siddiqi and Rüsch (2006) (described in Chapter 2, section 2.8.3 and 2.8.4).

Genomic DNA extraction

Genomic DNA was extracted from the culture of *M. tuberculosis* colonies by a method described by Somerville *et al.,* (2005), a loop full of *M. tuberculosis* colonies was suspended in 400 µl of 10 mM Tris–HCl, 1 mM EDTA with pH 8 buffer. It was heated for 20 minutes at 80°C for killing all *M.*

tuberculosis. Then lysozyme (1 mg/ml) was added, and incubated for 2 hours at 37°C. This was followed by addition of proteinase K (0.2 mg/ml) and 10% sodium dodecyl sulfate (SDS) (1.1%), and tubes were vortexed and incubated at 65°C for 20 minutes with continuous shaking. After incubation, a mixture of N-acetyl-N, N, N-trimethyl ammonium bromide (CTAB) [40 mM] and NaCl (0.1 M) was added, and then added NaCl (0.6 M) immediately. The mixture vortexed until the suspension turned milky, and incubated at 65°C for 10 minutes. A 750 µl chloroform-isoamy lalcohol (24:1) was then added, again vortexed and then centrifuged at 13,000 rpm in a microcentrifuge for 5 minutes at room temperature. The genomic DNA was then precipitated with 70% ethanol and re-suspended in 30 µl TE buffer. Finally, DNA quality and concentration were determined by spectrophotometer at the optical density of 260 nm and 280 nm (described in chapter 2, section 2.8.5).

PCR amplification

The PCR amplification of the first line drug target genes of *M. tuberculosis* was done by using gene specific primers, the details presented in table 4.1. These primers were also used during sequencing. The PCR reactions were conducted in volume of 25 µL final reaction mix containing: 2.5 µl of 10x PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.001% gelatin), 0.5 µl of 0.2 mM dNTPs, 0.75 µl of 25 mM magnesium chloride solution, 1.25 µl each of the 10 µM primers (forward and reverse primers), 0.125 µl of 2 U AmpliTag Gold polymerase (Perkin Elmer, USA), 1.25 µl of DMSO, 2 µl of genomic DNA, and 15.3 µl of double distilled water. The amplification was done by programming the thermocycler of Eppendorf™ up on the following condition: 95°C for 3 minutes for initial denaturation; followed by 40 cycles of denaturation at 95°C for 1 minute, annealing ranges from 55°C to 65°C for 30 second or 1 minute (summarized in table 4.1 for each gene), and extension at 72°C for 30 seconds. The final extension was at 72°C for 5 minutes. The PCR amplified products were examined on 1.5 % agarose gel electrophoresis using 100 base pairs DNA ladder.

Table 4-1: Primers that were used for PCR amplification and DNA sequencing of drug target genes for analysis of mutation in M. isolates

Gene		Primer Sequence (5'→3')	Position	Annealing	Amplicon Size (bp)	Reference
rpoB	Forward	TCGCCGCGATCAAGGAGT	981	65°C (30 sec)	272	Homolka *et al.,* 2010
	Reverse	GTGCACGTCGCGGACCTCCA	1253			
katG	Forward	TCGGCGATGAGCGTTACAGC	614	65°C (30 sec)	542	Zhao *et al.,* 2005
	Reverse	CCCGCAGCGAGAGGTCAGTGG	1156			
fabG1-inhA	Forward	CCTCGCTGCCCAGAAAGGGA	-168	55°C (1 min)	229	Talenti *et al.,* 1997
	Reverse	ATCCCCCGGTTTCCTCCGGT	61			
rpsL	Forward	CGGCGGGTATTGTGGTTGCTCGTG	-203	55°C (1 min)	800	Khan *et al.,* 2013
	Reverse	CCTCCAGGGCGGGTTTGACATTG	597			
Rrs	Forward	CTTATGTCCAGGGCTTCA	1202	55°C (1 min)	363	Khan *et al.,* 2013
	Reverse	CAGTTGGGGCGTTTTC	1565			
embB	Forward	TGGACGGGCGGGGCTCAAT	725	65°C (30 sec)	575	Khan *et al.,* 2013
	Reverse	CCAGCGCCGCCGGTGTGAGC	1300			
pncA	Forward	GCTGGTCATGTTCGCGATCG	-104	60 °C (30 sec)	708	Koivula *et al.,* 2004
	Reverse	CGCTCCACCGCCGCCAACAG	604			

Finally, EXOSAP cleanup of PCR products for sequencing was performed upon the following condition: 5 µl PCR products were mixed with 1µl exonuclease and 1µl alkaline phosphatase and then the mix was then placed in a thermal cycler with the hot lid off. The cycles were performed for 30 min at 37°C and 15 min at 80°C (Khan *et al.,* 2013). Followed by a Sephadex cleanup of sequence-PCR products as described in chapter 2 section 2.8.6.

DNA sequencing and analysis

The resulting products were sequenced with their gene specific forward and reverse primers using Big dye- terminator kit and ABI Prism 3500lL Genetic Analyzer (Applied Biosystems, USA). The sequencing data obtained from the ABI3730XL DNA analyzer were imported into SeqScape® software version 2.7 (Applied Biosystems, Foster City, CA) and consensus sequences generated. The SeqScape® was used for DNA sequence comparisons and mutations were detected in the respective gene by comparing with the reference *M. tuberculosis* strain H37Rv sequence.

Data analysis

All patient related information collected, phenotypic drug profiles and drug target genes mutation data were compiled. All data were entered, cleared and categorized as necessary. Descriptive statistics were computed to calculate the frequency and percentage of the socio-demographic, TB related conditions, phenotypic drug profiles and mutations identified from drug target genes data using SPSS version 23 statistical package software (SPSS Inc., Chicago, IL).

4.4 Result

Socio-demographic and TB related conditions

Out of 226 isolates processed for DNA extraction, only 209 (92.5%) isolates had enough DNA for sequencing for study of gene mutation, but the remaining 17 (7.5%) isolates did not have enough DNA for sequencing, and among the excluded isolates, there was one MDR-TB isolate from previously treated cases that had an additional resistance to STR drug. Although socio-demographic and TB related conditions were describe in chapter 3, we tried to described the important variables of the 209 cases. Of thse 209 cases, 124 (59.3%) were males, and majority 94 (45.0%) of the participants were in the age group of 24-34 years. Married individuals accounted for the majority 124 (59.3%) the cases. A total of 148 (70.8%) cases were previously treated cases. Of these, 134 (64.1%) were relapse cases and the remaining 14 (35.9%) case were treatment failure and defaulter cases. One hundred eighty-one (86.6%) cases were smeared positive, and 123 (58.9%) cases were also TB/HIV co-infected. Seventy-nine (37.8%) cases had an antibiotic treatment history and more than one-third, 28 (35.4%) cases interrupted antibiotic treatment for more than one times. Moreover, 41 (19.6%) of the participants were drinking alcohol and 26 (12.4%) also were smoking cigarettes frequently (Table 4.2).

Table 4-2: Socio-demographic and TB related conditions of MDR-TB suspected cases and MDR-TB confirmed cases, Addis Ababa, January, 2017(n=209)

Variable	All MDR-TB suspected Cases Number (%) (n=209)	MDR-TB confirmed Cases Number (%) (n=88)
Sex		
Male	124 (59.3)	37 (42.0)
Female	85 (40.7)	51 (58.0)
Age Group		
15-24	25 (12.0)	4 (4.5)
25-34	94 (45.0)	53 (60.2)
35-44	62 (29.7)	22 (25.0)
45-54	19 (9.1)	6 (6.8)
Above 54	9 (4.3)	3 (3.4)
Marital Status		
Married	124 (59.3)	53 (60.2)
Unmarried	75 (35.9)	31 (35.2)
Divorced	7 (3.3)	2 (2.3)
Widow	3 (1.4)	2 (2.3)
Living Region		
AA	193 (92.3)	82 (93.2)
Amhara	1 (0.5)	0 (0)
Dire Dawa	2 (1.0)	2 (2.3)
Oromia	11 (5.3)	4 (4.5)
SNNPR	2 (1.0)	0 (0)
Residence		
Rural	12 (5.7)	4 (4.5)
Urban	197 (94.3)	84 (95.5)
Previously TB infected		
No	61 (29.2)	16 (18.2)
Yes	148 (70.8)	72 (81.8)
Treatment history of previously TB infected cases		
No	0 (0)	0 (0)
Yes	148 (100)	72 (100)
Treatment interruption previously TB treated cases		
No	126 (85.1)	62 (86.1)
Yes	22 (14.9)	10 (13.9)
TB Treatment History		
New	61 (29.2)	16 (18.2)
Previously treated	148 (70.8)	72 (81.8)
Previously treated cases (n=148)		
Defaulter	5 (3.4)	3 (4.2)
Relapse	134 (90.5)	62 (86.1)
Treatment Failure	9 (6.1)	7 (9.7)
AFB Results for Sputum		
Negative	28 (13.4)	2 (2.3)
Positive	181 (86.6)	86 (97.7)
HIV Status		
Positive	123 (58.9)	70 (79.5)
Negative	86 (41.1)	18 (20.5)
Antibiotic treatment history		
No	130 (62.2)	45 (51.1)
Yes	79 (37.8)	43 (48.9)
Antibiotic treatment interruption		
No	51 (64.6)	28 (65.1)
Yes	28 (35.4)	15 (34.9)
Alcohol drinking frequently		
No	168 (80.4)	60 (68.2)
Yes	41 (19.6)	28 (31.8)
Alcohol drinking during treatment		
No	32 (78.0)	22 (78.6)
Yes	9 (22.0)	6 (21.4)
Cigarettes Smoking		
No	183 (87.6)	72 (821.8
Yes	26 (12.4)	16 (18.2)

SNNPR: Southern Nations, Nationalities, and Peoples' Region

Phenotypic Drug susceptibility (DST) Results

Out of 209 *M. tuberculosis isolates*, 122 (58.4%) isolates were resistant to at least one of five first-line anti-TB drugs (RIF, INH, PZA, EMB, and STR). The proportion of resistance to INH, STR, RIF, EMB and PZA were 109 (52.2%), 93 (44.5%) 88 (42.1%) 72 (34.4%), and 70 (33.5%) respectively. Moreover, the overall prevalence of MDR-TB was 88 (42.1%), and the prevalence among previously treated and new cases were found to be 72/148 (48.6%) and 16/61 (26.2%) respectively. Furthermore, additional resistant to STR, PZA and EMB was observed in 75 (85.2%), 65 (73.9%) and 62 (70.5%) MDR-TB Isolates respectively, of the MDR-TB isolates, 52 (59.1%) Isolates were resistance to all first-line anti-TB drugs (RIF, INH, PZA, EMB, and STR). A significant multi-drug-resistance rate was observed among smear positive 86 (97.7%) cases, and more than half of the isolates, 123 (58.9%) were TB/HIV co-infected cases (Figure 4.1).

Analysis of Gene Mutations

A total of 209 isolates had sequencing results for determination of mutations associated with drug target genes of rpoB, katG, fabG1-inhA promotor region, embB, pncA, rpsL, and rrs. The sequencing results were found from 148 previously treated and 61 new cases.

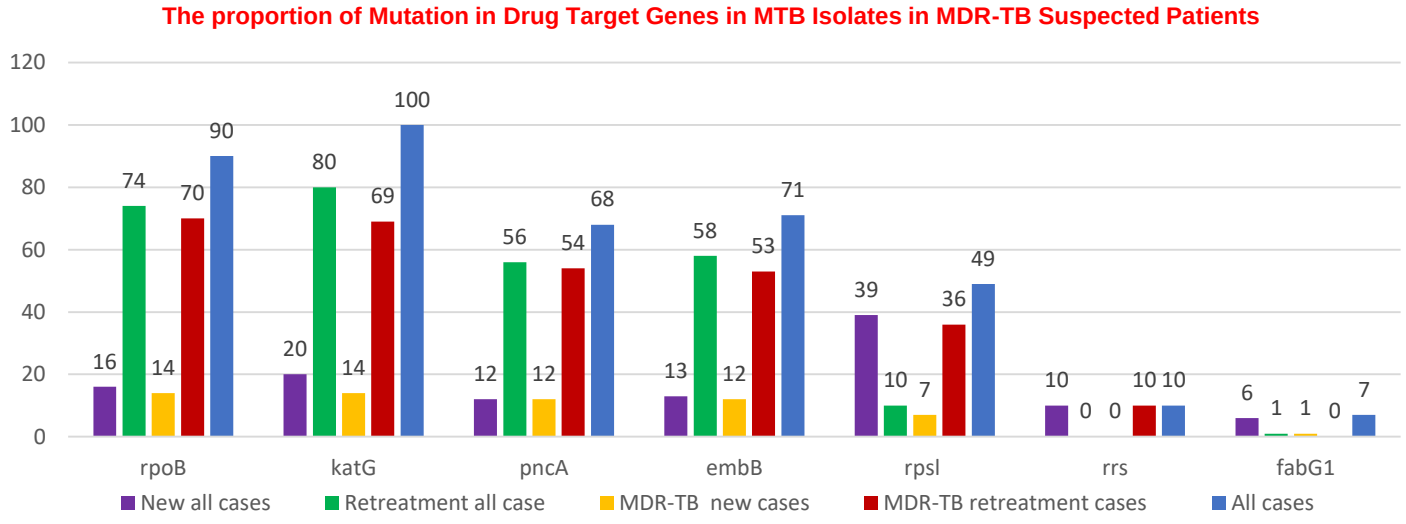


Figure 4-1: the proportion of mutation in drug target genes in MTB isolates among previously treated and new TB cases, and MDR-TB patients Addis Ababa, January, 2017 (n=209)

All (phenotypic drug resistant and susceptible) isolates were checked for mutation at the genes that target for Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), Ethambutol (EMB), and Streptomycin (STR) drugs summary of the result shown in figure 4.2.

Detection of mutations in rpoB gene

The 81bp hypervariable the rifampicin resistance determining region (RRDR) of rpoB gene was amplified using forward and reverse primers. Ninety (43.1%) isolates had the mutations in the RRDR of rpoB, and among phenotypic RIF resistance isolates 84/88 (95.5%) isolates had rpoB mutations, while 5 isolates from phenotypic susceptible isolates showed a mutations at codon 529, 533 and 538, and another 1 isolate had a silent mutation at codon 531. The sensitivity of mutation analysis in rpoB gene was 95.5% with 95.1% specificity. The major single-nucleotide polymorphisms (SNPs) for rifampicin resistance were found between codons 513 and 533 in the RRDR of rpoB gene and 88 (97.8%) isolates showed single mutation at rifampicin resistance determining region of rpoB gene. Furthermore, 85 (94.4%) rpoB mutant strains had an additional mutation in katG gene and 89 (98.9%) had an additional mutation any drug target genes [kat, fabG1, pncA, embB, rpsL, rrs genes] (Table 4.7).

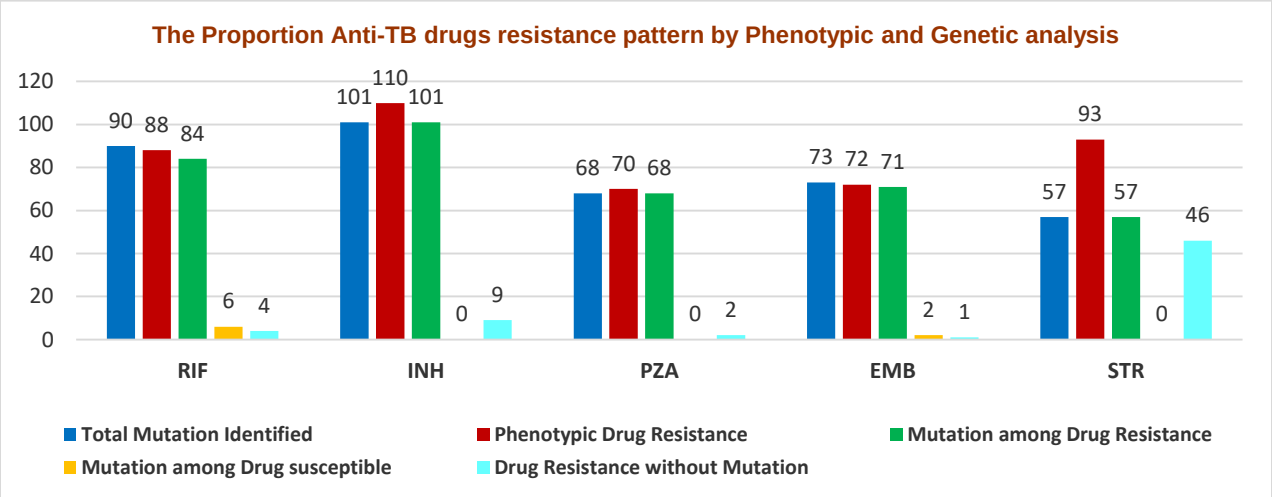


Figure 4-2: The Proportion Anti-TB drugs resistance pattern by Phenotypic and Genetic analysis, Addis Ababa, January, 2017 (n=209)

The highest 61 (67.8%) nucleotide substitution occurred at codon 531 of the rpoB gene, where 2 (S531L, TCG →TTG, and S531W, TCG → TGG), and different other types of single nucleotide substitutions were identified, predominantly replacing serine with leucine in 58 (95.1%) isolates and serine substituting by tryptophan in 3 (4.9%) isolates. Moreover, the second highest nucleotide changes were observed in 15 (16.7%) isolates at codon 526 (H526Y [CAC to TAC], H526S [CAC to AGC], H526D [CAC to GAC], and H526L [CAC→ CTC]). Of these, majority 10/15 (66.7%) of the isolates had a mutation at H526Y (CAC →TAC).

Table 4-3: Drug resistance mutations of rpoB gene of *M. tuberculosis* among new and previous treated TB cases

Codon	Nucleotide Change(s)	Amino acid Change(s)	All cases (n=90) n (%)	Previously treated cases (n=74) n (%)	New cases (n=16) n (%)	MDR-TB Cases New (n=14)	MDR-TB Cases Previously treated (n=70)
513 insA	A inserted	Q513 insA	1(1.1)	1 (1.4)			1 (1.4)
513	CAA→ CCA	Q to P	3(3.3)	-	3 (18.8)	3 (21.4)	-
516	GAC→GTC	D to V	4(4.4)	3 (4.1)	1 (6.3)	1 (7.1)	3 (4.3)
522	TCG→ TTG	S to L	1(1.1)	1 (1.4)			1 (1.4)
526	CAC→TAC	H to Y	9 (10.0)	7 (9.5)	2 (12.5)	2 (14.3)	7 (10.0)
526, 531**	CAC→TAC, TCG→TTG,	H to Y, S to L,	1 (1.1)	1(1.4)	-	-	1 (1.4)
526	CAC→ GAC	H to D	2 (2.2)	2 (2.7)	-	-	2 (2.9)
526	CAC→ CTC	H to L	1 (1.1)	1 (1.4)	-	-	1 (1.4)
526	CAC→AAC	H to S	1 (1.1)	1 (1.4)	-	-	1 (1.4)
526	CAC→AGC	H to S	1 (1.1)	1 (1.4)	-	-	1 (1.4)
531	TCG→TTG	S to L	57 (63.3)	49 (66.2)	8 (50.0)	7 (50.0)	49 (65.3)
531, 548**	TCG→TTG, CGC→CGG	S to L, R to R*	1 (1.1)		1 (6.3)	1 (7.1)	-
531	TCG→TGG	S to W	3(3.3)	3 (4.1)	-	-	3(4.3)
531	TCG→TCC	S to S*	1(1.1)	1 (1.4)	-	-	-
533	CTG→ CCG	L to P	2(2.2)	2 (2.7)	-	-	-
538	CTG→ CCG	L to P	1(1.1)	1 (1.4)	-	-	-
538	CTG→ GTG	L to V	1(1.1)	-	1(6.3)	-	

* Silent mutation; ** Gene that has multiple mutations;

In addition the remaining mutations were found at codons D516V, Q513P, L533P, and L538P/V with isolates number 4 (4.4%), 3 (3.3%), 2 (2.2%), and 2 (2.2%), respectively. Another more 1

(1.1%) isolate showed insertion of a single nucleotide A at codon 513. Furthermore, 2 (2.2%) isolates had multiple mutations at codons H526Y (CAC → TACC) and S531L (TCG →TTG) and at L531S (TTG →TCG) and R548 (CGC → CGG) [silent mutation], as well as 1 (1.1%) isolate had a silent mutation at codon S531 [TCG→TCC] (Table 4.3).

Detection of mutations in katG and fabG1-inhA genes

In order to determine the frequency of mutations in INH resistant isolates, the commonly affected reported genes of katG, the promoter region of fabG1 and inhA were sequenced and analyzed for identification of mutations, and we found mutation in katG gene and promoter region of the fabG1-inhA in 107 (51.2%) isolates. Of these, 100 (93.5%) isolates had a mutation in katG and the remaining 7 (6.5%) had in the fabG1-inhA promoter region conferring INH-resistance. However among phenotypic INH resistance isolates, 101/110 (91.8%) isolates had the mutations in katG gene and promoter regions of the fabG1-inhA, and 9 (8.1%) phenotypic INH resistance isolates did not show mutation either in katG or fabG1-inhA promoter region. So that the overall sensitivity and specificity were 91.8% and 100% respectively.

Moreover, multiple mutations were identified in 6 isolates, in which mutations were present at codon 315 in katG and at the -8. -15, 14, and 28 positions of the promoter of the fabG1-inhA operon in addition to this, we found a mutation due to insertion of A in katG at codon 221 (nucleotide position between 661-662). Besides, as shown in table 4.7, 85 (85.0%) katG mutant strains had an additional mutation in rpoB gene, and 95 (95%) katG mutant strains had an additional mutation in any drug target genes (rpoB, fabG1, pncA, embB, rpsL, rrs genes). The most common nucleotide substitutions occurred at codon S315T of the katG gene, and the highest nucleotide substitutions were AGC to ACC (serine to threonine) in 98/100 (98.0%) of the isolates. Additionally, a mutation in the fabG-inhA promoter region was C to T transition at a point -15 in 4/7 (57.2%) isolates. Furthermore, 1 (1%) isolate had a double mutation at codons S315T and T275A (ACC → GCC) [Table 4.4].

Table 4-4: Drug resistance mutation of katG and fabG(mabA)-inhA promotor region of *M. tuberculosis* among new and previous treated TB cases

Gene	Codon	Nucleotide Change(s)	Amino acid Change(s)	All cases (n=107) n (%)	Previously treated cases (n=86), n (%)	New cases (n=21), n (%)	MDR-TB Cases (n=89)	
							New cases n (%)	Previously treated n (%)
KatG gene	**315**	A**G**C→ACC	S to T	97 (97)	78 (97.5)	19 (95.0)	14 (100)	67 (97.2)
	315	AGC→AG**A**	S to H	1 (1.0)	1 (1.25)	-	-	1 (1.4)
	334		E to S	1 (1.0)	-	1 (5.0)	-	
	221	661-662 insertion G	**Fs**	1 (1.0)	1 (1.25)	-		1 (1.4)
fabG1-inhA	**-15**	C- →T	C- to T	4 (57.1)	4 (66.1)	-	-	3 (60.0)
	-8	T- → C	T- to C	1 (14.3)	1 (16.7)	-	-	1 (20.0)
	14	T→G	T to G	1 (14.3)	1 (16.7)	-	-	1 (20.0)
	28	G→C	G to C	1 (14.3)	-	1 (100)	1 (100)	-

Fs: frameshift

Detection of mutations in embB gene

In this study, mutation analysis was done in embB gene, and a total of 73 (34.9%) isolates had a mutation at a different part of embB gene. Of 72 phenotypic EMB resistance isolates, 71 (98.6%) Isolates were identified as mutants, and the remaining 1 (1.4%) isolate had no mutation at embB gene; in another hand, 2 (2.7%) mutants were not phenotypic EMB resistance. Therefore, the sensitivity and specificity were found to be 98.6% and 98.5% respectively. As shown in table 4.5 a total of 66 (90.4 %) embB mutants were MDR-TB strains. Of which 54 (81.8%) strains were from previously treated patients. Sixty-eight (93.2%) embB mutants had addition mutation in katG gene, while 60 (82.2%) mutants had addition mutation rpoB gene. In addition, most 71 (97.3%) of the embB mutants had an additional mutation at any drug target genes [rpoB, katG, fabG1, pncA, rpsL, rrs genes] (Table 4.7).

The highest proportion of mutation was observed at codon 306 in 48 (65.8%) isolates, that predominantly replacing methionine with isoleucine (ATG → ATC, ATG →ATC) in 25/48 (52.1%) isolates, and methionine substituting by valine (ATG → GTA)in 21/48 (43.8%) isolates.

Table 4-5: Drug resistance mutations of embB gene of *M. tuberculosis* among new and previous treated TB cases

Codon	Nucleotide Change(s)	Amino acid Change(s)	All cases (n=73) n (%)	Previously treated cases (n=59) n (%)	New cases (n=14) n (%)	MDR-TB Cases New (n=12)	MDR-TB Cases Previously treated (n=54)
306	ATG→ ATA	M to I*	17 (23.3)	11 (18.6)	6 (42.9)	6 (50.0)	10 (18.9)
306, 347*	ATG→ ATA, 1039 del A	M to I, fs*	1 (1.4)	1 (1.7)			1 (1.9)
306, 406*	ATG→ ATA, GGC→GCC	M to I, G to A*	1 (1.4)	1 (1.7)			1 (1.9)
306, 406*	ATG→ ATA, GGC→GAC	M to I, G to D*	1 (1.4)	1 (1.7)			1 (1.9)
306	ATG→ ATC	M to I	5 (6.8)	3 (5.1)	2 (14.3)	1 (8.3)	3 (5.7)
306	ATG→ GTA	M to V	21 (28.8)	21 (35.6)			21 (39.6)
306	ATG→ CTA	M to L	2 (2.7)	1 (1.7)	1 (7.1)	1 (8.3)	1 (1.9)
311	GAC→GGC	D to G	1 (1.4)	1 (1.7)			
316, 354*	ATG→AGG, GAT→ GCT	M to R, D to A*	1 (1.4)	1 (1.7)			1 (1.9)
328, 35A*	GAT→ CAT GAT→ GCT	D to H, D to A*	1 (1.4)	1 (1.7)			1 (1.9)
332	TGG→GGG	W to G	1 (1.4)	1 (1.7)			1 (1.9)
347, 402*	AGT→TGT, 1204 del C	S to C, fs*	1 (1.4)	1 (1.7)			1 (1.9)
378, 406*	GAG→GCG, GGC→GAC	E to A, G to D*	1 (1.4)	1 (1.7)			1 (1.9)
406	GGC→GCC	G to A	13 (17.8)	10 (16.9)	3 (21.4)	3 (25.0)	10 (18.9)
409	GCG→CCG	A to P	1 (1.4)	1(1.7)			
347	1039 del A	Fs	4 (5.5)	2 (3.4)	2 (14.3)	1 (8.3)	2 (3.8)
368	1101-1102 ins G	Fs	1 (1.4)	1 (1.7)			1 (1.9)

* Gene that has multiple mutations, **fs: frameshift, del: deletion, ins: insertion**

Moreover, 13 (17.8%) strains had a mutation at codon G406A (GGC → GCC), and frameshift mutation was found at codon 347 with deletion of a single nucleotide A in 4 (5.5%) isolates, and another 1 isolates had frameshift mutation due to insertion of G between codon 367 and 368. Furthermore, multiple mutations was observed in 7 (9.6%) strains at codons M306I andG406D, M306I andG406A, M306I and 347 (deletion of A), M316Rand D354A, D328Hand D354A, S347C and402 [deletion of C), and E378A and G406D (Table 4.5).

Detection of mutations in pncA gene

In this study, a total of 68 (30.1%) isolates had a mutation in pncA gene, and all [68 (100%)] isolates were phenotypic PZA resistance, however among PZA resistance (n=70), 2 (2.9%) isolates did not show any mutation in pncA gene, in addition, majority 66 (97.1%) of mutants were MDR-TB strains. The overall correlation between phenotypic drug susceptibility and genotypic tests result was found to be 99.0%, with 97.1% sensitivity and 100% specificity. Further analyses of the mutation in PncA gene indicated that 65 (95.6%) pncA mutant strains had addition mutation in rpoB and katG gene, and all [68 (100%)] mutants had an additional mutation at any drug target genes such as rpoB, katG, fabG1, embB, rpsL, or rrs genes. Moreover, the highest rate of mutation was found between position 192–193 (codon 64 and 65) due to insertion of a single nucleotide A in 19 (27.9%) isolates, and the second highest rate mutation was identified from 7 (10.3%) isolates at codon 130 (V130G/M, GTG → GGG/ATG) [Table 4.6].

Moreover, as a summary data shown in table 4.6, 8 mutations were identified from 20 (29.4%) isolates at codon T76P (ACT → CCT), A102V (GCA→GTA) and V139A (GTG → GCG), Y41Stop (TGG→TGA), Y103H (TAC→ CAC), 108 (G108E [GGA → GAA], G108A [GGA →GCA]), and L182W (TTG→TGG). Two (2.9%) isolates had A to G transition at -11 of the promoter region of pncA. Furthermore, we found a frameshift mutations between position 300 and 301, and 529 and 530 due to insertion of GC and A respectively, and another mutation was found between position 380 and 388 due to deletion of AGGTCGATG. In addition to this, multiple mutations was identified from a single isolate at codon G108A (GGA→GCC) and deletion of G at position 547.

Detection of mutations in rpsL and rrs genes

A total of 57 (27.3%) isolates had the mutations in rpsL and or rrs genes, and the highest proportion of mutation was found in rpsL gene in 49 (86.0%) strains, and all 57 (100%) isolates were phenotypic STR resistance, and majority 50 (87.7%) of the mutants were MDR-TB strains.

Table 4-6: Drug resistance mutations of pncA gene of *M. tuberculosis* among new and previous treated TB cases

Codon	Nucleotide Change(s)	Amino acid Change(s)	All cases (n=68) n (%)	Previously treated cases (n=56) n (%)	New cases (n=12) n (%)	MDR-TB Cases New (n=14)	MDR-TB Cases Previously treated (n=54)
64-65	192-193 Ins A	Fs	19 (27.9)	19 (33.9)	-	-	19 (35.2)
130	GTG→ GGG	V to G	5 (7.4)	5 (8.9)	-	-	5 (9.3)
76	ACT→ CCT	T to P	4 (5.9)	2 (3.6)	2 (16.7)	2 (16.7)	2 (3.7)
102	GCA→GTA	A to V	4 (5.9)	3 (5.4)	1 (8.3)	1 (8.3)	3 (5.6)
139	GTG→GCG	V to A	4 (5.9)	2 (3.6)	2 (16.7)	2 (16.7)	2 (3.7)
41	TAC→TAG	Y to STOP	2 (2.9)	2 (3.6)	-	-	2 (3.7)
103	TAC→ CAC	Y to H	2 (2.9)	1 (1.8)	1 (8.3)	1 (8.3)	1 (1.9)
130	GTG→ATG	V to M	2 (2.9)	2 (3.6)	-	-	
44	GTC → GGC	V to G	1 (1.5)	1 (1.8)	-	-	1(1.9)
46	GCA→ ACA	A to T	1 (1.5)	1 (1.8)	-	-	1 (1.9)
48	AAG→GAG	K to E	1 (1.5)		1 (8.3)	1 (8.3)	-
57	CAC→TAC	H to Y	1 (1.5)	1 (1.8)	-	-	1 (1.9)
6	ATC→ACC	I to T	1 (1.5)	1 (1.8)	-	-	1 (1.9)
72	TGC →CGC	C to R	1 (1.5)	1 (1.8)	-	-	1 (1.9)
78	GGC→AGC	G to S	1 (1.5)	1 (1.8)	-	-	-
100-101	300-301 ins GC	Fs	1 (1.5)	1 (1.8)	-	-	-
108	GGA→GCC	G to A	1 (1.5)	1 (1.8)	-	-	1 (1.9)
108, 183*	GGA→GAA, 547 del G	G to E, fs*	1 (1.5)	1 (1.8)	-	-	1 (1.9)
119	TGG→CGG	W to R	1 (1.5)	1 (1.8)	-	-	1 (1.9)
119	TGG→TGA	W to Stop	1 (1.5)	1 (1.8)	-	-	1 (1.9)
119	TGG→ TGT	W to C	1 (1.5)	1 (1.8)	-	-	1 (1.9)
12	GAC→ GCC	D to A	1 (1.5)	1 (1.8)	-	-	1 (1.9)
182	TTG→ TGG	L to W	2 (2.9)	2 (3.6)			2 (3.7)
-11	A→ G	A to G	2 (2.9)	2 (3.6)			2 (3.7)
7	GTC→ GGC	V to G	1 (1.5)		1 (8.3)	1(8.3)	-
127	380-388 del AGGTCGATG	Fs	1 (1.5)	1 (1.8)	-	-	1 (1.9)
129	GAT→ AAT	D to N	1 (1.5)		1 (8.3)	1(8.3)	-
138	TGT→TGG	C to W	1 (1.5)	1 (1.8)			1 (1.9)
155	GTG→ GCG	V to A	1 (1.5)		1 (8.3)	1(8.3)	-
160	ACA→ CCA	T to P	1 (1.5)	1 (1.8)			1 (1.9)
177	529-530 ins A	Fs	1 (1.5)		1 (8.3)	1(8.3)	-
180	GTC → TTC	V to F	1 (1.5)		1 (8.3)	1(8.3)	-

* Gene that has multiple mutations, fs: frameshift; ins; insertion, del: deletion

However, among phenotypic STR resistant isolates (n = 93), 46 (38.7%) strains had no mutation in rpsL or rrs gene. Thus, the overall correlation between phenotypic and genotypic test results was found to be 82.8%, and the sensitivity and specificity were 61.4% and 100% respectively.

Table 4-7: Mutation Pattern in *M. tuberculosis* complex isolates among previously treated and new MDR-TB suspected cases Addis Ababa, January, 2017 (n=209)

Pattern of Mutation in Drug target Genes	All Case n () (n=209)	All Case (n=209)		MDR-TB Cases (n=88)	
		Previously treated (n=148) n (%)	New Cases (n=61) n (%)	Previously treated (n=72) n ()%	New Cases (n=16) n (%)
Single Mutation					
rpoB	5 (2.4)	2 (1.4)	2 (3.3)	-	-
KatG	5 (2.4)	3 (2.0)	2 (3.3)	1 (1.4)	-
EMB	2 (1.0)	1 (0.7)	1 (1.6)	-	-
rpsL	2 (1.0)	1 (0.7)	1 (1.6)	-	-
Any Mutation	110 (52.6)	87 (58.8)	23 (37.7)	71 (98.6)	14 (87.5)
rpoB	90 (43.1)	74 (50.0)	16 (26.2)	70 (97.2)	14 (87.5)
KatG	100 (47.8)	80 (54.1)	20 (32.8)	69 (95.8)	14 (87.5)
fabG-inhA	7 (3.3)	6 (4.1)	1 (1.6)	5 (6.9)	1 (6.3)
embB	73 (34.9)	59 (39.9)	14 (23.0)	54 (75.0)	12 (75.0)
pncA	68 (32.5)	56 (37.8)	12 (19.7)	54 (75.0)	12 (75.0)
rpsL	49 (23.4)	39 (26.4)	10 (16.4)	36 (50.0)	7 (43.8)
Rrs	10 (4.8)	10 (6.8)		10 (13.9)	
Multiple Mutation					
rpoB + KatG+ fabG-inhA + embB + pncA + rpsL	2 (1.0)	1(0.7)	1(1.6)	1 (1.4)	1()
rpoB + KatG+ embB + pncA + rpsL + rrs	2 (1.0)	1(0.7)	1(1.6)	1 (1.4)	1()
rpoB + KatG+ embB + pncA + rpsL	26 (12.5)	20 (13.5)	6 (9.8)	20 (27.8)	6 (37.5)
rpoB + KatG+ embB + pncA + rrs	3(1.4)	3 (2.0)		3(4.2)	
rpoB + KatG+ fabG-inhA + embB + pncA +rrs	1 (0.5)	1(0.7)		1 (1.4)	
rpoB + KatG+ fabG-inhA + embB + pncA	2(1.0)	2(1.4)		2(2.8)	
rpoB + KatG+ embB + pncA	19 (9.1)	17(11.5)	2 (3.3)	17 (23.6)	2 (12.5)
rpoB + KatG+ embB + rpsL	5 (2.4)	3 (2.0)	2 (3.3)	3 (4.2)	2 (12.5)
rpoB + KatG+ pncA + rpsL	4 (1.9)	4 (2.7)		3 (4.2)	
rpoB + KatG+ pncA	6 (2.9)	4 (2.7)	2 (3.3)	3 (4.2)	2 (12.5)
rpoB + KatG+ embB	5 (2.4)	3 (2.0)	2 (3.3)	3 (4.2)	2 (12.5)
rpoB + KatG+ rpsL	5 (2.4)	5(3.4)		5 (6.9)	
rpoB + KatG	5 (2.4)	4 (2.7)	1 (1.6)	4 (5.6)	1 (6.3)
rpoB + embB + pncA + rrs	1 (0.5)	1(0.7)		1 (1.4)	
KatG+ embB + pncA	1 (0.5)		1(1.6)		1 (6.3)
KatG+ embB + rpsL	1 (0.5)	1(0.7)			
KatG+ embB	3 (1.4)	2 (1.4)	1 (1.6)		
KatG+ rpsL	2 (1.0)	1(0.7)	1(1.6)		1 (6.3)
KatG+ pncA	1 (0.5)	1(0.7)			

The finding from rpsL gene showed that most 43 (87.8%) of isolates had a mutation at codon K88R/T (AAG→ AGG/ACG), moreover, the frequency of mutations at codon K43R (AAG→ AGG) was found to be 6 (12.2%). Furthermore, mutations at rrs gene were identified in 10 MDR-TB isolates at position 514 (A → C), 517 (C→T), 613 (A → C), 891 (G → A), 906 (A → G), and 1010 (A → C) as shown in table 4.8. As well as multiple mutations were found in rpsl and rrs genes in 2 isolates at codon K88R (AAG→ AGG) in rpsL and 891 (G → A) in rrs, and K88T (AAG→ ACG) in rpsL and 613 (A → C) in rrs. In addition to this, 1 isolate had multiple mutation in rpsL gene at codon K43R (AAG→ AGG) and K88R (AAG→ AGG). Besides, 43 (87.8%) rpsL mutant isolates had an additional mutation in rpoB and katG gene and out of 49 rpsL mutants, 47 (100%) isolates had an additional mutation in any drug target genes such as rpoB, katG, fabG1, embB, pncA, or rrs gene shown in table 4.7 above.

Table 4-8: Drug resistance mutations of rpsL and rrs gnes of *M. tuberculosis* among new and previous treated TB cases

Gene	Codon	Nucleotide Change(s)	Amino acid Change(s)	All cases (n=107) n (%)	Previously treated cases (n=86), n (%)	New cases (n=21), n (%)	MDR-TB Cases (n=87)	
							New cases n (%)	Previously treated n (%)
rpsL gene	88*	AAG→AGG	K to R*	22(44.9)	16(41)	6(60)	2(28.6)	15(42.9)
	88**	AAG→ACG	K to T**	21(42.9)	21(53.9)		2(28.6)	18(51.4)
	43	AAG→AGG	K to R	6(12.2)	2(5.1)	4(40.0)	3(42.9)	2(5.7)
	88 and 43***	AAG→AGG	K to R and K to R***	1(2.0)	1(2.0)			1(2.0)
rrs gene****	514 ¥	A → C	-	2(20)	2(20)	-	-	2(20)
	517	C → T	-	2(20)	2(20)	-	-	2(20)
	631**, ¥	A → C**	-	1(10)	1(10)	-	-	1(10)
	891 *, ¥	G → A*	-	2(20)	2(20)	-	-	2(20)
	906 ¥	A → G		2(20)	2(20)			2(20)
	1010¥	A →C	-	1(10)	1(10)	-	-	1(10)

* Multiple mutations was found in rpSL (K88R) and rrs (891, G → A); ** Multiple mutations was found in rpSL (K88T) and rrs (631, A → C) *** Gene that has multiple mutations, ¥ numbers indicated nucleotide position

4.5 Discussion

This study revealed that the prevalence and pattern of drug resistance associated mutations in RIF, INH, PZA, EMB and STR resistant *M. tuberculosis* strains circulating in Addis Ababa Ethiopia. Moreover, to our knowledge, although several studies were done for analysis of drug

resistance using molecular techniques, our study is the first study done by sequence for analysis of drug target genes of *M. tuberculosis* strains circulating in Ethiopia. With regard to this, the information provided from our study would be a preliminary sequencing data for rpoB, KatG, embB, pnncA, rpsL, rrs and fabG1-inhA promoter region genes from strains collected from MDR-TB suspected Patients in Ethiopia.

In this study, the major single-nucleotide polymorphisms (SNPs) for rifampicin resistance were found between codons 513 and 533 in the RRDR of rpoB gene, and 97.8% isolates showed a single mutation in RRDR of rpoB gene. In supporting our finding, studies showed that rifampicin resistance mutation was found within a hotspot between codon 507 and 533 in RRDR (Mohajeri *et al.,* 2015, Racheal *et al.,* 2015). In another finding, about 5% rifampicin resistance isolates did not have any mutation in the rpoB gene. This resistance could be due to intrinsic drug resistance mechanism in which it is attributed to its unique cell wall properties, including the presence of mycolic acids, which are high-molecular-weight α-alkyl, β-hydroxy fatty acids covalently attached to arabinogalactan, and which constitute a very hydrophobic barrier responsible for resistance to certain antibiotics (Karakousis *et al.,* 2004). On the other hand, about 7% of the mutant strains that had the mutations at codon L533P L538P and L538V of rpoB genes were RIF susceptible. This situation might be due to the existence of *M. tuberculosis* strains with borderline susceptibility or the presence of a mixture of susceptible and resistant strains simultaneously (Van *et al.,* 2005).

The highest rate of mutation in rpoB gene was found at codon 531 with 67% isolates and more than 95% of the mutation was substituting serine to leucine (TCG to TTG). This finding was comparable with several studies from Pakistan, Thailand, Georgia and Singapore (Qazi *et al.,* 2014, Htike *et al.,* 2014, Shubladze *et al.,* 2013, Lee *et al.,* 2005**).** Moreover, the second highest rate of mutation was found at codon 526 in 16.5% of the isolates, this is also comparable with reports from various countries (Ramaswamy and Musser, 1998, Prammananan *et al.,* 2008). In another finding, 5% of the isolates showed a mutation at codon 516 (GAC to GTC) with

substitution aspartate to valine, it was in consistent with a study from Georgia (5.2%) (Shubladze *et al.,* 2013), however studies from Pakistan (21%), (Qazi *et al.,* 2014), Singapore (12%) (Lee *et al.,* 2005**)** and Thailand (9.1%) (Prammananan *et al.,* 2008) showed the highest proportion of mutation at codon 516 of rpoB gene. It might be due to different selection pressure operation and genotypes of *M. tuberculosis* predominantly circulating in the different geographical locations.

Moreover, a mutation at codon L538 P/V (CTG → CCG and GTG) was found in 2.2% of isolates that were 5 codons away from 81 bp RRDR of rpoB, it is worth noting that the two mutations was revealed in this study, indicating that there is considerable diversity in rpoB gene which has implication in development of resistance however unaddressed by the molecular diagnostic approaches undermine the outcome. Therefore, in countries like Ethiopia where GeneXpert MTB/RIF assay is used as screening diagnostic tool, it is important to screen MDR-TB suspected patient using culture for those who have RIF susceptible result from GeneXpert MTB/RIF Assay. Furthermore, a similar finding was reported from Thailand that showed a mutation at codon 538 mutation,, it indicates that there is important diversity in rpoB gene mutation (Htike *et al.,* 2014). On the other hand, there is another report that shows not all mutations in rpoB are associated with RIF resistance, such as mutation at L533P is not associated with RIF resistance, and it was identified in RIF-susceptible strains (Andres *et al.,* 2014). So as we identified 2.2% mutation at codon L533P, our finding caution us against relying solely on the molecular methods such as GeneXpert MTB/RIF Assay for diagnosis of RIF resistance, and suggesting that the molecular methods might not reliable as phenotypic DST. So it is important to confirm MDR-TB suspected patient using culture for those who have RIF resistant TB result from GeneXpert MTB/RIF Assay.

Likewise, mutations were identified in katG gene and promoter regions of the fabG1 (mabA)-inhA in more than half (51.2%) of the isolates, and more than 93% of the mutation was found in katG gene. Moreover, more than 8% of the phenotypic INH resistant isolates did not have any mutation in katG gene and promoter regions of the fabG-inhA. This situation might be due to the intrinsic

drug resistance mechanism of the bacilli (Karakousis *et al.,* 2004), and or due to a mutation in another gene such as ahpC regulatory region (Piatek *et al.,* 2000, Khan *et al.,* 2013). The significant mutation was found at codon 315 in katG gene with highest nucleotide substitution AGC to ACC (serine to threonine) in 97% of the strains. This mutation has been the most widely reported from several studies, and our finding was in consistent with study done by Hillemann *et al.,* (97.2%) (Hillemann *et al.,* 2005), and other studies from Kazakhstan and Pakistan were comparable with our study (Kozhamkulov *et al.,* 2011, Khan *et al.,* 2013).

Multiple mutations were found in katG codon 315 and the promoter region of the fabG1-inhA promotor region at the positions -8 and -15 in 6% of the isolates, the finding was also comparable with a study done in Kazakhstan (Kozhamkulov *et al.,* 2011). Moreover, about 99% of MDR-TB strains had a point of mutation in a katG gene, and the highest frequency of mutation was found at codon S315T. It is comparable with study dome by Shubladze *et al.,* (2013), and it suggests that mutations in katG at codon 315 confer a survival advantage on INH-resistant isolates that would be more likely to survive in TB patients, to be transmitted, and become an MDR strain. This finding is in agreement with the study done by Hazbon *et al.* (2006). Thus, the mutation at codon 315 in katG could be used as a biomarker for a screening of MDR-TB in suspected patients.

Mutation in pncA gene is the main mechanisms for pyrazinamide resistance in MTB. Our study revealed that more than 30% of the isolates had the mutations in pncA gene, and the highest proportion (27.9%) of mutation was found between codon 64 and 65 caused by insertion of A. There are reports that indicate the presence of frameshift mutation in this position (Bhuju *et al.,* 2013, Lemaitre *et al.,* 1999) and also a study done by Lemaitre *et al.,* showed that similar nucleotide insertion between codon 64 and 65. But the higher percentage in our study could be due to a different geographical distribution of these isolates and a large number of MDR-TB strains. Moreover, our study found out that mutations in pncA gene was highly diverse and distributed throughout the gene were observed in more than 25 codons that causing amino acid

substitutions. Our finding is similar to several previous studies done by Hirano *et al.* (1997), Chang *et al.* (2011) and Cui *et al.* (2013). The findings from our and previous studies help us to conclude that there is no clear hotspot for pncA mutation. So it might be a challenge for the development of a molecular method for detection of PZA resistance in MTB.

However, when we compare it with the phenotypic results, the correlation was found to be more than 97%. This finding is also comparable with several studies in different countries such as in Japan (97%) (Hirano *et al.,* 1998), South Korea (97%) (Lee *et al.,* 2001), and slightly higher than South Africa (92%) (Mphahlele *et al.,* 2008), and China (91%) (Hou *et al.,* 2000). But there are other studies that reported much lower than our finding; 46% in Brazil (Bhuju *et al.,* 2013), and 67% in South Africa (Bishop *et al.,* 2001). Our higher concordance might be because of large proportion of MDR-TB strains in our study. Moreover, an interesting observation is that mutation in PncA gene indicated that all the pncA mutants had an additional mutation in any other drug resistance determining region of gene such as rpoB, katG, fabG1, embB, rpsL, or rrs gene. This situation might favor the survival advantage of this group or it could be a predisposing factor for the other drug genes mutation.

In addition, 34.9% of the isolates had a mutation in embB gene, of which more than 91% of the mutants were MDR-TB strains. However, 1.5% EMB resistance isolates did not have any mutation in the embB gene. A similar, study also showed that EMB-resistant isolates did not harbor mutations in any part of the embB gene, suggesting alternative mechanisms of EMB resistance (Karakousis, 2009). On the other hand, about 3% EMB susceptible isolates had the mutations at an embB gene, similarly, the situation could be due to the existence of MTB strains with borderline susceptibility or the presence of a mixture of susceptible and resistant strains simultaneously (Van *et al.,* 2005). The most common mutation in embB gene was observed at codon 306 in more than 66% of the isolates, the finding of our study is comparable with several other studies that showed

the proportion of mutation at codon 306 was reported between 50% and 70% (Khan *et al.,* 2013, Ramaswamy and Musser, 1998, Ramaswamy *et al.,* 2000, Telenti *et al.,* 1997b).

Moreover, the major mutation at codon 306 was predominately substitution of methionine to isoleucine and valine, and it was found mainly in MDR-TB isolates with more than 95.8% of the mutants. Our study suggests that there might be an association between mutation at codon 306 and rpoB and katG genes, so a mutation at codon 306 might be a predisposing factor for the development of MDR-TB. In supporting our assumption, the study revealed that mutation at codon 306 was associated with resistance to other anti-TB drugs, which further indicated that patients with embB resistant would be at risk for the development of MDR-TB (Safi *et al.,* 2008). As Hazbón *et al.,* suggested that the mutation at codon 306 prevent the synergistic effect of the combination of multiple anti-TB drugs (Hazbón *et al.,* 2005), TB patients infected with codon 306 mutants should be carefully monitored for MDR-TB (Safi *et al.,* 2008). Furthermore, another mutation in embB was also found at codon D311G, M316R, D328H, W332G, S347C, E378A, G406A, and A409P. But the second highest rate of mutation was identified at codon 406 (GGC→GCC) with 18% isolates. Our finding was also comparable with studies done by Ramaswamy *et al.* (2000) and Zhao *et al.* (2015).

Moreover, our finding revealed that the point of mutations in rpsL and rrs genes were found in 52.1% and 10.6% of isolates respectively, this is closely consistent with a study done by Heym *et al.,* (1994) (52% and 8%). However, among STR resistance, about 50% of the isolates did not have any mutation in both genes, this indicates strains resistant to streptomycin that lack mutations in either of these two genes may have other mechanisms of resistance (Karakousis *et al.,* 2004). In addition, some low-level STR resistance seems to be caused by increased efflux as efflux pump inhibitors caused increased sensitivity to STR (Spies *et al.,* 2008). Besides, this finding shows the performance of molecular technique is not good for diagnosis STR resistant TB. However, as culture test method requires several weeks to identify the resistance pattern,

this approach may have clinical utility for rapid diagnosis of STR resistant TB until new reliable and accurate method developed.

Likewise, a mutation in the rpsL gene was identified at codon 43 (12.2%) and 88 (87.8%) by substituting lysine to arginine and threonine, and they are the most common genetic alterations present in STR resistant isolates. Comparing with other studies, our finding showed that mutation at codon K43R was lower (12.2%) than reported data from Brazil (25%) (Spies *et al.,* 2011), India (42.9%) (Yadav *et al.,* 2013), and Singapore (80.4%) (Sun *et al.,* 2010) this difference could be due to different geographical distribution of these isolates, but there are comparable reports from Africa, Asia, and Latin America (Tekwu *et al.,* 2014, Cuevas-Co´rdoba *et al.,* 2013, Sun *et al.,* 2010, Spies *et al.,* 2008). The frequency of mutation at codon 88 was in agreement with the data reported from Singapore, and Brazil (Sun *et al.,* 2010, Spies *et al.,* 2008). Moreover, multiple mutations genes were found in two MDR-TB strains at codon K88T in rpsl and position 631 and 891 in rrs, mutations in these loci have very rarely been observed to occur concurrently (Cuevas-Co´rdoba *et al.,* 2013, Sun *et al.,* 2010). This may suggest that mutational alterations in either rrs or rpsL may alleviate the need for the modification of the other gene (Jagielski *et al.,* 2014b).

Furthermore, about 53% of the Isolates had any mutation, and the frequencies and occurrence of multiple mutations was found to be more than 87% of the various drug target genes, and almost all strains were MDR-TB. It suggests that the presence of multiple mutations might be predisposing factors for the development of drug-resistant TB especially MDR-TB, our assumption also supported by another study (Safi *et al.,* 2008). Moreover, our study revealed that the pattern of drug target gene mutation is higher in previously treated cases which indicates that previously treated cases are more at risk to develop drug-resistant TB than new cases. In addition, when comparing the performance of phenotypic drug susceptibility and genotypic tests, the overall correlation was found between 82 and 99%, however the sensitivity was found to be 61.4% for STR, 91.8% (INH), 95.5% (RIF), 98.6% (EMB) and 99.0% (PZA). Various studies showed

different performance in both methods (Campbell *et al.,* 2011, Safi *et al.,* 2008). This variation might be because of different geographical distribution of the strains and pattern of resistance, however, we suggest further study to investigate the main factors for variations among studies in different countries.

4.6 Conclusion

In this study, the highest rate of point mutations among drug-resistant TB isolates were found at codon 531, 315, 306, 65, and 88 in rpoB, katG, embB, pncA, and rpsl genes respectively. Moreover, this study revealed that majority of the MDR-TB cases had a mutation at codon 306 and the mutation at codon 306 was found to be an important molecular indicator of EMB resistance and MDR-TB. In addition, the mutations were found at 5 codons far way from 81 bp RRDR at codon 538 and also at 533 with RIF susceptible in RRDR, so it is very important to screen MDR-TB suspected patient using other methods than GeneXpert. Our study revealed that the patterns of resistance and diversity of mutations in drug target genes of MTB in Ethiopia were similar to the majority of reports globally. This may have important implications for the roll-out of rapid molecular tests to identify drug-resistant MTB strains, but, there should be a system for continuous monitoring of the patterns of drug-resistance associated mutations.

Furthermore, as our study was the first study done by using sequencing methods for detection of drug resistance in *M. tuberculosis* complex isolates in Ethiopia, this study pointed out that there are discrepancy results between phenotypic drug susceptibility test and molecular analysis of mutation responsible for drug resistance. Our study suggests that further studies should be done using sequencing technique to investigate factors associated with resistance mechanisms. In conclusion, the prevalence of MDT-TB was high in both previously treated and newly infected cases. Therefore, strengthening TB control program, the DOTS strategy and laboratories for early diagnosis and proper management of TB/MDR-TB in the country are crucial in order to prevent and control the emergence and transmission of MDR-TB in the population.

Chapter 5: Publication III

5. Genotyping of Mycobacterium tuberculosis isolates among MDR-TB suspected patients from Addis Ababa, Ethiopia

Submitted to Journal of BMC infectious diseases

5.1 Abstract

Background: Multidrug drug-resistant tuberculosis (MDR-TB) is a major health problem and a result of unsuccessful TB control programs. Thus, understanding the genetic diversity of the circulating population of *M. tuberculosis* can enhance the prevention of TB/MDR-TB infections. The aim of this study was to investigate the population structure and transmission dynamics of *M. tuberculosis.*

Methods: A cross-sectional study was conducted in Addis Ababa, between June 2015 and December 2016. Drug susceptibility test was done for Rifampicin, Isoniazid, Pyrazinamide, Ethambutol, and Streptomycin, and sequencing was performed for respective drug target genes. Genotyping analyses were performed by using 24-loci MIRU-VNTR and spoligotyping techniques.

Results: A total of 167 isolates were classified according to the phylogenetic classification of *M. tuberculosis* isolates using both 24-loci MIRU-VNTR profiles and spoligotyping patterns, 157 (94.0%) were classified into previously described lineages as follows: 50(29.9%) strains were Dehli/CAS, 44 (26.3%) TUR, 25 (15.0%) H37Rv like, 17 (10.2%) TUR_Ethiopia, 14 (8.4%) Haarlem, 4 (2.4%) Ural, 1 (0.6%) LAM (Latin American Mediterranean), 1 (0.6%) X-type, and 1 (0.6%) EIA. However, 10 (6.0%) of the isolates were appeared to be as previously undefined lineages. The overall prevalence of MDR-TB was 76 (45.5%), and the high prevalence was observed in TUR and Delhi/CAS families with 35 (46.1%), and 27 (35.5%) respectively. A total of 103 (61.7%) strains were grouped in 19 clusters ranging in size from 2 to 26 strains, resulting in a recent transmission index (RTI) of 50.3%, indicating a high rate of recent transmission.

Conclusions: This study revealed a highly diverse *M. tuberculosis* population structure, and the most predominant lineages were Dehli/CAS family and TUR with the higher prevalence of multi-drug-resistance TB. The high rate of recent transmission of MDR-TB indicates that there is weak health system for prevention and control MDR-TB/TB in Ethiopia. This highlights the importance of strengthening health system to improve TB control program at large.

Key words: *Mycobacterium tuberculosis*, Genotyping, Transmission Dynamics, MDR-TB,

5.2 Introduction

Tuberculosis (TB) continues as a major health problem globally. It causes a disease among millions of people each year and it is the second leading cause of death from an infectious disease worldwide. In 2016 there were about 10.4 million TB cases, and more than 1.3 million TB deaths and about 85% of TB deaths occurred in African and South-East Asia Region. Moreover, drug-resistant (DR) TB is one of the major global threat, and according to WHO report, in 2016, there were about half a million multidrug-resistant TB (MDR-TB) patients, MDR-TB is defined as resistance to both isoniazid and rifampicin, the two most effective first-line drugs, and the global prevalence of MDR-TB was 4.1% and 19% among new and previously treated cases respectively. Almost half (47%) of these cases were from India, China and the Russian (WHO, 2017). In addition to this, another report showed that the prevalence of MDR-TB is also high in Sub-Saharan countries especially among previously treated TB cases (Asres *et al.,* 2013).

Ethiopia is one of the 30 high burden countries for TB, TB/HIV and MDR-TB globally, and a disease of tuberculosis remains one of the leading causes of mortality in the country. According to 2017 WHO report, the prevalence of MDR-TB was 14% among previously treated and among new cases was 2.7%, in addition to this, 8% of TB cases were TB/HIV co-infected patients (WHO, 2017). However several studies done in Ethiopia showed that the prevalence of MDR-TB was varid from place to place such as 31.4% in Jimma (Kedir *et al.,* 2015), 46.3% in Addis Ababa (Dereje et. al., 2012). 5% in Northwest Ethiopia. (Tessema et. al., 2012). The emergence of drug resistance to MDR-TB is a major public health threat for the populations of resource-limited setting and become a significant obstacle for TB control program. In addition to this, a high prevalence of infectious diseases and limited access to health care facilities making worst the consequence of MDR-TB. Moreover, poor treatment outcomes, longer treatment time, treatment costs, and many more complications make MDR-TB a more complex disease in the population of the sub-Saharan region (WHO, 2010b).

Genotypic variations among *M. tuberculosis* strains as well as the existence of human genetic polymorphism linked to TB have resulted in the changing relationship between *M. tuberculosis* and the human host. Such changes have complicated TB control efforts (Dye and Williams, 2010). Data on strain diversity of mycobacterial isolates is important to understand the transmission dynamics and phylogeographical distribution of dominant circulating strains of *M. tuberculosis*. Molecular typing of *M. tuberculosis* complex (MTBC) has gained increasing acceptance as a powerful tool to facilitate our understanding of epidemiology of TB (Van Soolingen 2001). In the last decades, a large number of different molecular methods based on DNA fingerprints have been developed. The usefulness of these methods has been demonstrated primarily as epidemiological markers to discriminate the pathogen at the genus, species, and subspecies level. The level of strain differentiation is of crucial importance for the study of transmission dynamics, determining whether the infection is caused by single strain or by multiple strain and if recurrence of the disease is due to treatment failure or infection with new strain of *M. tuberculosis* (Kontsevaya *et al.,* 2011).

In addition genotyping methods have been applied extensively in the epidemiological study of TB worldwide (Sharma and Mohan 2004. These studies have been based on the assumption that patients with genotyping clustered strains are epidemiologically linked and represent recent transmissions. In contrast, patients infected with different types of strains are not considered indicative of recent transmission. Genetic markers need to be both sufficiently polymorphic to distinguish unrelated strains and stable enough to identify isolates of the same strains. Furthermore, the typing methods employed must be reproducible, discriminatory and easy to perform.

Spoligotyping is the second most widely used method for *M. tuberculosis* complex genotype after IS*6110*-based fingerprinting. It is based on the presence or absence of a set of target sequences in the direct repeat (DR) locus in the *M. tuberculosis* complex genome that in combination with

Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats (MIRU-VNTRs) typing has been used to replace typing via restriction fragment length polymorphisms (RFLP) based on the insertion sequence IS*6110*. It has been shown to be a valuable alternative to IS*6110* (Supply *et al.,* 2001). An optimized 24-loci MIRUVNTR typing scheme has been proposed as an international standard (Thong-On *et al.,* 2010). In addition to their use for tracing TB transmission at the strain level, MIRU-VNTR markers are also phylogenetically more informative, especially in the 24- locus format and can therefore be used to predict grouping into strain lineage (Oelemann *et al.,* 2011). Currently, Whole genome sequencing (WGS) analysis classifies MTBC into seven main lineages; lineages 2, 3, 4 and 7 belong to the evolutionary modern group and are considered more recently diversified compared to the ancient lineages of 1, 5 and 6 (Coscolla and Gagneux, 2014).

MTBC and the human host have been a co-evolutionary relationship for several centuries and the origin of *MTB* was Africa and co-evolved into modern lineages and then migrated from Africa to other part of the world (Galagan 2014). The lineage distribution among cases caused by *M. tuberculosis* exerts distinct geographical associations worldwide (Hershberg *et al.,* 2008, Coscolla and Gagneux, 2014). While lineages 1 (The Philippines and Rim of Indian Ocean) and 3 (India and East Africa) are prevalent in East Africa, Central, South- and South-East Asia, and lineages 2 (East Asia) and 4 (Europe, America and Africa) are the most widely distributed worldwide. Lineages 5 and 6, which are also known as *M. africanum* West Africa 1 and West Africa 2, respectively, are localized in West Africa (De Jong *et al.,* 2010). Lineage 7 is a *M. tuberculosis* lineage recently discovered in north-western Ethiopia (Firdessa *et al.,* 2013, Comas *et al.,* 2015).

Since members of *M. tuberculosis* complex (MTC) have highly conserved genomes, high definition tools are needed to reveal the subtle changes within the infecting mycobacterial population. The accurate identification of different strains at individual and population level

provides an insight into disease dynamics which are essential in clinical diagnostics, treatment and population control strategies. Mycobacterial Interspersed Repetitive Units-variable number of tandem repeat (MIRU-VNTR) analysis use variations in a copy of repeats in highly variable regions of the MTC genome to shows changes in the genome over relatively shorter time periods (Supply *et al.,* 2006). In order to understand the epidemiology of TB globally as well as locally, the use of molecular typing methods has become a vital not only for understanding TB genetic diversity and population structure of MTBC but also for conducting monitoring and evaluating of TB control program and for understanding of TB epidemiology (Banu *et al.,* 2015). Currently, spoligotyping and MIRU-VNTR typing have been used as molecular genotyping methods for analysis of MTB genotyping (Brudey *et al.,* 2006).

Although several studies done on the characterization of MTBC isolates in Ethiopia, the majority of the studies used a spoligotyping technique (Mulalem *et al.,* 2010, Amare *et al.,* 2012, Adane *et al.,* 2012, Garedew *et al.,* 2013, Mulugeta *et al.,* 2014), spoligotyping is PCR-based reverse hybridization blotting technique based on polymorphism in the presence or absence of "43 spacers" in the Direct Repeat (DR) locus of MTBC genome. Even if spoligotyping is quick and suitable genotyping method it has less discriminatory power than MIRU-VNTR typing method, and recent studies have pointed out that sometimes phylogenetically unrelated MTBC strains tend to have same spoligotype patterns as a result of convergent evolution or due to independent mutational changes (Fenner *et al.,* 2011). However, MIRU-VNTR typing method has better discriminatory power and is less prone to homoplasy. It is a better tool if it will be used along with spoligotyping (Oelemann *et al.,* 2007). Though, MIRU-VNTR typing method along with spoligotyping has better discriminatory power and is less prone to homoplasy, there are only a few studies done in Ethiopia which have used combined analysis of spoligotyping and MIRU-VNTR typing method (Tessema *et al.,* 2013, Biadglegn *et al.,* 2015, Solomon *et al.,* 2015), and all of the studies were done in Amhara region, that cover limited geographical area of the country

which is different from our study sites and participants type (MDR-TB suspected cases) and socio-economic activities where high influx of people to the metropolis from all corner of the country.

As result, there is little information about the genetic characteristics of the isolates driving the epidemic in Ethiopia, and MDR-TB continues as a major public health problem. A better knowledge of the molecular characteristics of *M. tuberculosis* complex isolates could contribute to better understanding of the transmission dynamics of the disease within the country, and can guide interventions to control the MDR-TB epidemic. So analyzing genetic diversity of drug-resistant TB isolates would help to determine the population structure of the circulating strains and transmission dynamics of *M. tuberculosis* strains for prevention and control of MDR-TB/TB. Therefore, the aim of this study was to investigate the population structure and transmission dynamics of *M. tuberculosis* among MDR-TB suspected cases from Ethiopia using high resolution MIRU-VNTR 24-loci combined with spoligotyping.

5.3 Materials and Methods

Study Setting and Design

A cross-sectional survey was conducted between June 2015 and December 2016 in selected health facilities found in Addis Ababa, Ethiopia. Data and sputum specimens were collected from study participants visiting the health facilities during the study period. Sample size was calculated using single population proportion formula considering the assumptions that at 95% confidence level with 5% precision and z value of 1.96 (Barlett *et al.,* 2001), and 17.8% prevalence of MDR-TB among previously treated cases (WHO, 2015). Thus considering 10% nonresponse rate, the final sample size calculated was 248. Since Addis Ababa Health research and Laboratory services, Saint Peter hospital and Teklehiamnot health center are a referral diagnostic sites for an MDR-TB suspected patient in Addis Ababa, they were selected as study sites. Volunteer an MDR-TB suspected patients who visited the health facilities during the study period were included

as study participants. MDR TB suspected patients who are seriously ill or unconscious, below the age of 12 years old and not willing to participate in the study were excluded from the study.

Sputum Specimens collection and storage

Sputum specimens from MDR-TB suspected patients were collected from the selected health facilities for the study. A volume of 5 to 10 ml sputum specimen produced by a deep cough from the patient was collected into a sterile wide mouth 50 ml falcon tube. All specimens were stored at 2-8^{o}C at collection sites until transported to Ethiopian Public Health Institute National TB Reference laboratory using cold chain.

Laboratory Analysis and Drug Susceptibility Test (DST)

Sputum specimens were analyzed using Ziehl-Neelsen Methods (WHO, 1998), GeneXpert MTB/RIF assay (Barnard *et al.,* (2012), Culture (LJ and MGIT) methods as described previously by Kent and Kubical (1985) and Siddiqi and Rüsch (2006). All positive cultures were further confirmed using MPT64 antigen detection methods (Capilia TB) (ECDC, 2016). Phenotypic drug susceptibility test for rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) were performed with the Bactec MGIT 960 method as described by Siddiqi and Rüsch (2006), (described in chapter 2, section 2.8.3 and 2.8.4).

Extraction of DNA

Genomic DNA was extracted from the culture of *M. tuberculosis* colonies by a method described by Somerville *et al.* (2005) as described in chapter 2 section 2.8.5.

Genotyping Analysis

Spoligotyping

Spoligotyping was performed for 204 *M. tuberculosis* isolates using a standard method to detect the presence or absence of 43 spacers as described by Kamerbeek *et al.,* (1997). Briefly, Direct Repeat (DR) region of MTB strains was amplified using specific primers, and the PCR amplicons

were subsequently hybridized to a set of 43 different immobilized DR spacers covalently bound to a membrane, and then incubated with a streptavidin-peroxidase conjugate. After incubation, the hybridization signals were detected using chemiluminescence system. Spoligotypes were reported using an octal code in which the 43-digit binary representing the 43 spacers by yielding a 15-digit octal designation (Dale *et al.,* 2001) as described in chapter 2 section 2.8.8.3.

MIRU-VNTR typing

A total of 167 isolates were genotyped using a 24-loci MIRU-VNTR genotyping technique as described by Supply *et al.,* (2006). Briefly, 24 loci were amplified by using the MIRU-VNTR typing kit (Genoscreen, Lille, France), and the PCR products were analyzed using the Rox-labeled MapMarker 1,000 size standard for mix 1–4, and mix 6–8, and 1500 size standard for mix 5 (BioVentures, Inc., Murfreesboro, VT) by ABI 3730XL sequencer with 16 capillaries (Applied Biosystems, Foster City, CA). Analysis of the PCR fragments and assignment of the various VNTR alleles were done using the GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA) as described in chapter 2 section 2.8.8.4.

Interpretation of Spoligotyping and MIRU-VNTR results

The spoligotyping results were compared to the international database SITVIT with an updated version of the published SpolDB4.0 database (Brudey *et al.,* 2006), and MIRU results were compared with the MIRU-VNTR plus Database (http://www.miru-vntrplus.org) to determine MTB strain lineages and relatedness. Cluster analysis was performed using the Bionumerics software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated by using the unweighted pair group method with arithmetic averages (UPGMA), and minimum spanning tree (MST) analysis was done based on MIRU-VNTR typing data using the categorical coefficient (described in chapter 2 section 2.8.8.5).

Gene Mutation Analysis

PCR Amplification for Sequencing

The PCR amplification for target genes was performed by a method described by Khan *et al.,* (2013) as described in chapter 4 section 4.3.4.

DNA sequencing and analysis

The resulting products were sequenced with their gene specific forward and reverse primers using Big dye- terminator kit and ABI Prism 3500lL Genetic Analyzer (Applied Biosystems, USA). The sequencing data obtained from the ABI3730XL DNA analyzer were analyzed SeqScape® software version 2.7 (Applied Biosystems, Foster City, CA) as described in chapter 2, section 2.8.7.

Data analysis

All data were entered, cleared and analyzed using SPSS version 23 statistical package software (SPSS Inc., Chicago, IL). Bivariate analyses were carried out for categorical variables, and for determining of potential risk factors, odds ratios (OR) and 95% confidence intervals (CI) were calculated by using logistic regression analysis for socio-demographic, TB related conditions (previous TB treatment, smoking, antibiotic treatment, alcohol drinking, HIV status), and drug-resistant pattern microbiological, *M. tuberculosis* lineage. A p-value of 0.05 was used as the cut-off point for statistical significance

Ethical Considerations

Ethical approval was obtained from Research and Ethical Review Committee of the Addis Ababa University. The Study subjects were told about the benefit of being tested. Besides, a written and or oral informed consent was taken from each study participant. Data and specimens were collected and analyzed using codes for confidentiality purpose.

5.4 Results

A total of 204 DNA specimens were obtained from MDR-TB suspected strains for genotyping analysis, and analyses were carried out using MIRU-VNTR 24-loci and spoligotyping Methods.

Out of 204 isolates, 37 were excluded from the final analysis, of which 21 isolates had no PCR amplicon at two or more loci, and 13 isolates had no any PCR amplicon, and the remaining 3 isolates were identified as a mixture of two independent strains during MIRU-VNTR typing. An occasional lack of PCR amplification of some loci has been reported in previous studies (Supply *et al.,* 2006). The possible reasons might be due to chromosomal deletion, nucleotide polymorphisms in the sequences complementary to PCR primers or insufficient DNA quality (Tessema *et al.,* 2013). A mixture of two independent strains was also defined by the presence of double alleles at two or more loci (Supply *et al.,* 2006). Isolates with no PCR amplicon at only one locus were treated as missing data at the respective loci and included into the analysis. Finally a total of 167 isolates had valid genotyping data for the genotyping analysis.

Socio-demographic characteristics of the study participants

As a total of 167 valid results obtained from genotyping analysis, 167 MDR-TB suspected cases were included for analysis data. Of these, 101 (60.5%) of cases were males, and majority 73 (43.7%) of the cases were in the age group of 25-34 years with an average age of 34.5 years, and most 155 (92.8%) of the study participants were living in urban. Married individuals accounted for the majority 100 (59.9%) of the cases, but 59 (35.3%) were never married at all. Majority 51 (30.5%) of the cases were self-employed, and followed by 46 (27.5%) private organization, 26 (15.6%) government organization, 25 (15.0%) daily laborer, and the remaining 19 (11.4) were unemployed (house wife and unemployed). Twenty- (90.2%) were relapse seven participants graduated from colleges, and 51 Fifty-one (30.5%) of the respondents attended high schools and followed by 44 (26.3%) elementary attendants and the remaining 45 (27.0%) did not attend any formal education (Table 5.1).

TB and TB related conditions among MDR-TB suspected case

A total of 143 (85.6%) cases were AFB positive, and 97 (58.1%) cases were TB/HIV co-infected. One hundred and twelve (67.1%) cases were previously treated cases that had a history of TB

treatment for more than a month in addition to this, 65 (38.9%) cases had a history of family member infected by TB. Moreover, among the previously treated cases (n=112), 101 (90.2%) were a relapse and the remaining 11 (.8%), were treatment failure and defaulter cases. In addition, 14 (12.5%) cases had discontinued anti-TB drug during treatment time. One hundred and thirty-eight (82.7%) case were visiting health facilities, and 39 (23.4%) cases were admitted to hospital. Sixty-four (38.3%) cases had an antibiotic treatment history, of which 23 (35.9%) cases interrupted antibiotic treatment for more than one times. Furthermore, 32 (19.2%) and 21 (12.6%) participants were alcohol drinkers and cigarettes smokers respectively (Table 5.1).

Population structure and cluster analysis

Spoligotyping

All TB isolates were subjected to 43-spacers spoligotyping, and the spoligotyping patterns of isolates were classified according to the SITVIT database, and 15 different spoligotypes and 12 orphan patterns were observed among the studied isolates. Of 167 *M. tuberculosis* isolates, 58 (34.7%) were T3-ETH strains, 26 (15.6%) CAS1-Kili strains, 23 (13.8%) CAS1-Delhi strains, and 13 (7.8%) T3. Eleven (7.2%) strains belonged to each H3 and T1 family while 3 (1.8%) isolates belonged to the T2 family, and 2 (1.2%) to the LAM3 family. The minor families observed in our study were belonged to the LAM9 1 (0.6%) H37Rv 1 (0.6%), LAM7-TUR 1 (0.6%), EAI8-MDG family 1 (0.6%) and H1 1 (0.6%). Another 2 (1.2%) isolates were not classified into families according to SpolDB4, but did have attributed ST numbers according to the SITVIT database, displayed an ambiguous result with T3 and T2. Moreover, 1 (0.6%) isolates displayed unknown pattern with no matches to any of the major clades present in the database. In addition, 12 (7.2%) isolates, comprising 12 spoligotyping patterns were orphans' strains. In another finding, 141 (84.4%) isolates were categorized into shared-types, and the remaining 26 strains exhibited unique SIT patterns.

Table 5-1: Socio-demographic and TB related conditions of MDR-TB suspected and MDR-TB confirmed cases among genotyping strains, Addis Ababa, January, 2017(n=167)

Variable	All MDR-TB suspected Cases Number (%) (n=167)	MDR-TB confirmed Cases Number (%) (n=76)
Sex		
Male	101(60.5)	32 (42.1)
Female	66 (39.5)	44 (57.9)
Age Group		
15-24	21 (12.6)	4 (5.3)
25-34	73 (43.7)	44 (57.9)
35-44	52 (31.1)	21 (27.6)
45-54	12 (7.2)	4 (5.3)
Above 54	9 (5.4)	3 (3.9)
Marital Status		
Married	100 (59.9)	46 (60.5)
Unmarried	59 (35.3)	26 (34.2)
Divorced	5 (3.0)	2 (2.6)
Widow	3 (1.8)	2 (2.6)
Living Region		
Addis Ababa	152 (91.0)	70 (92.1)
Out of Addis Ababa	15 (9.0)	6 (7.9)
Residence		
Rural	12 (7.2)	4 (5.3)
Urban	155 (92.8)	72 (94.7)
Family Previously TB infected		
No	102 (61.1)	55 (72.4)
Yes	65 (38.9)	21 (27.6)
Treatment history of previously TB cases		
No	0 (0)	0 (0)
Yes	112 (100)	60(100)
Treatment interruption previously TB treated cases		
No	98 (87.5)	52 (86.7)
Yes	14 (12.5)	8 (13.3)
TB Treatment History		
New	55 (32.9)	16 (21.1)
Previously treated	112 (67.1)	60 (78.9)
History of previously treated cases		
Defaulter	4 (3.6)	3 (5.0)
Relapse	100 (89.3)	50 (83.3)
Treatment Failure	8 (7.1)	6 (11.7)
AFB Results for Sputum		
Negative	24 (14.4)	2 (2.6)
Positive	143 (85.6)	74 (97.4)
HIV Status		
Positive	97 (58.1)	59 (77.6)
Negative	70 (41.9)	17 (22.4)
Antibiotic treatment history		
No	103 (61.7)	37 (48.7)
Yes	64 (38.3)	39 (51.3)
Antibiotic treatment interruption		
No	41 (64.1)	25 (61.5)
Yes	23 (35.9)	14 (38.5)
Alcohol drinking		
No	135 (80.8)	53 (69.7)
Yes	32 (19.2)	23 (30.3)
Alcohol drinking during treatment		
No	24 (75.0)	19 (82.6)
Yes	8 (25.0)	4 (17.4)
Cigarettes Smoking		
No	146 (87.4)	63 (82.9)
Yes	21 (12.6)	13 (17.1)
Health facility visiting		
No	29 (17.4)	7 (9.2)
Yes	138 (82.6)	69 (90.8)
Hospital admission		
No	128 (76.6)	46 (60.5)
Yes	39 (23.4)	30 (39.5)

T3-ETH family, represented the predominant pattern, accounting for 56 (39.7%) of clustered isolates in the study followed by CAS1-Kili with 26 (18.4%) and CAS1-Delhi 21 (14.9%) strains. Moreover, the transmission index rate was found to be 77.8% (Table 5.6).

MIRU-VNTR typing

Using 24-loci MIRU-VNTR genotyping, closely related patterns were observed between *M. tuberculosis* strains, and we were able to assign a lineage to 167 isolates with the 24-loci MIRU-VNTR pattern. Accordingly, 156 (93.4%) were classified into previously described lineages and observed 73 different 24-loci MIRU-VNTR patterns. Of 167 isolates, 50 (29.9%) were Dehli/CAS, 44 (26.3%) TUR, 25 (15.0%) H37Rv like, 17 (10.2%) TUR_Ethiopia, 13 (7.8%) Haarlem, 4 (2.4%) Ural, and 1 (0.6%) LAM (Latin American Mediterranean). However, the remaining 11 (6.6%) of the isolates were appeared to be previously undefined lineage. Regarding to clustering, 113 (67.7%) isolates were categorized into 19 shared-types ranging in size from 2 to 26 strains, and the remaining 54 (32.3%) strains showed a unique patterns. The largest 26 (23.0%) cluster is formed by the TUR genotype followed by Dehli/CAS genotype 21 (18.6%). Interestingly all TUR_Ethiopia-3 genotype was clustered in one group (n = 17). Moreover, a recent transmission index (RTI) was determined and found to be 77.8% (Table 5.6).

Combined Spoligotyping and MIRU-VNTR typing

As shown in figure 5.1, combined numerical analysis of spoligotyping and MIRU-VNTR data was done. Lineage assignation was performed by MIRU-VNTRplus best match labeling using 24 MIRU-VNTR and spoligotyping and using a human expert interpretation of spoligotype signatures. Accordingly, 167 isolates were assigned to a lineage using both the MIRU-VNTR 24-loci profiles

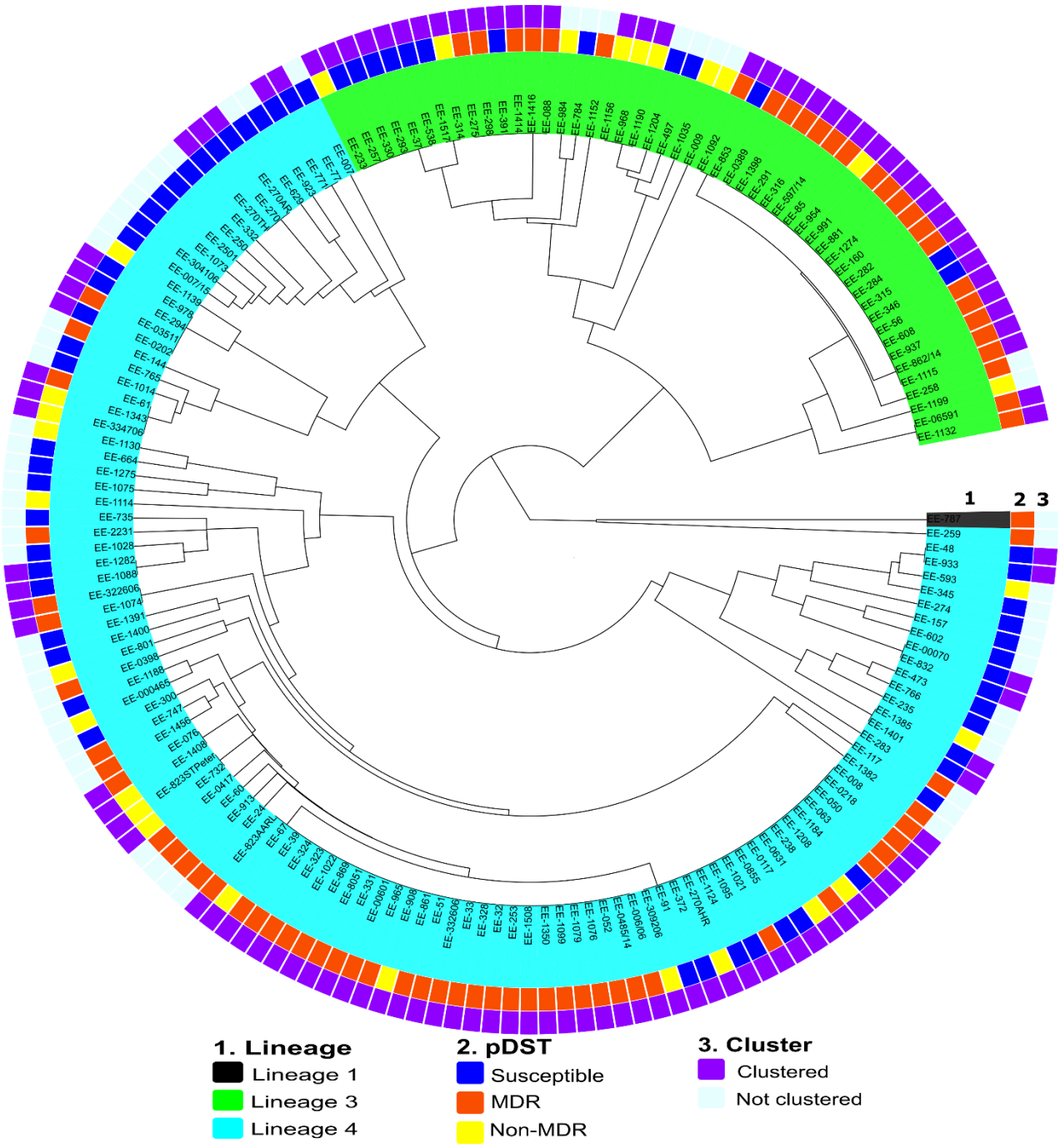


Figure 5-1: Radial UPGMA tree based on the copy numbers of MIRU-VNTR 24-loci and Spoligotyping, and it shows the pattern of drug resitance lineage and clustering of each genotype. The tree was calculated by using the MIRU-VNTR*plus* website

and spoligotyping patterns. According to the phylogenetic classification of 167 *M. tuberculosis* isolates, 157 (94.0%) were classified into previously described lineages as follows: 50 (29.9%) strains were Dehli/CAS, 44 (26.3%) TUR, 25 (15.0%) H37Rv like, 17 (10.2%) TUR_Ethiopia, 14

(8.4%) Haarlem, 4 (2.4%) Ural, 1 (0.6%) LAM, 1 (0.6%) X-type, and 1 (0.6%) EIA. However, 10 (6.0%) of the isolates were appear to be previously undefined lineage (Table 5.2).

Table 5-2: Distribution of genotyping strains by history of treatment (retreatment and new cases) and Muliti-drug resistance Pattern in *M. tuberculosis* complex isolates within January, 2017 (n=167)

	All cases			MDR-TB cases	
Lineage	**New**	**Retreatment**	**MDR-TB (n=76)**	**New**	**Retreatment**
Delhi/CAS (n=50)	14 (28.0%)	36 (72.0%)	27 (54.0%)	4 (14.8%)	23 (85.2%)
EAI (n=1)	0 (0.0%)	1(100.0%)	1 (100.0%)	0 (0.0%)	1(100.0%)
H37Rv-like (n=25)	10 (40.0%)	15 (60.0%)	3 (12.0%)	0 (0.0%)	3 (100.0%)
Haarlem (n=14)	8 (57.1%)	6 (42.9%)	0 (0.0%)	-	-
LAM (n=1)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	1(100.0%)
TUR (n=44)	12 (27.3%)	32 (72.7%)	35 (79.5%)	9 (25.7%)	26 (74.3%)
TUR_Ethiopia_3 (n=17)	4 (23.5%)	13 (76.5%)	6 (35.3%)	2 (33.3%)	4 (66.7%)
Undefined (n=10)	4 (40.0%)	6 (60.0%)	2 (20.0%)	0 (0.0%)	2 (100.0%)
URAL (n=4)	2 (50.0%)	2 (50.0%)	0 (0.0%)	-	-
X (n=1)	1(100.0%)	0 (0.0%)	1 (100.0%)	1(100.0%)	0 (0.0%)
Total (n=167)	55 (32.9%)	112 (67.1%)	**76 (45.5%)**	16 (21.1%)	60 (78.9%)

Distribution of drug resistance among various lineages

Among 167 *M. tuberculosis* isolates, the majority 104 (62.3%) of isolates were resistant to at least one of the five first-line anti TB drugs (RIF, INH, PZA, EMB, and STR). The overall prevalence of Multi-drug-resistant TB was 76 (45.5%). A higher multi-drug-resistance proportion was observed in TUR and Delhi/CAS with 35 (46.1%), and 27 (35.5%) isolates respectively, and among all first-line anti TB drugs resistant MDR-TB Isolates (n=37), most 31 (82.7%) of the isolates were from TUR and Delhi/CAS lineage and the remaining 6 (17.3%) were from H37Rv-like, X, EAI and undefined lineage group. Furthermore, among STR resistance group, high proportion of resistance were observed in TUR and Delhi/CAS lineage with 34 (41.5%), and 29 (35.4%) respectively, and 25 (39.7%) and Delhi/CAS lineage were resistance to EMB as well PZA resistance was found in 25 (39.7%) of TUR and 24 (40.7%) Delhi/CAS lineage. The pattern of multidrug resistance within a lineage was found to be 35 (79.5%), 27 (54.0%), 6 (35.3%), 3

(12.0%), 2 (20.0%) with TUR, Delhi/CAS, TUR_Ethiopia_3, H37Rv-like, undefined respectively. In addition to this, the lineages of EAI, LAM, and X, which represent by a single isolate, were MDR-TB strains (Table 5.3).

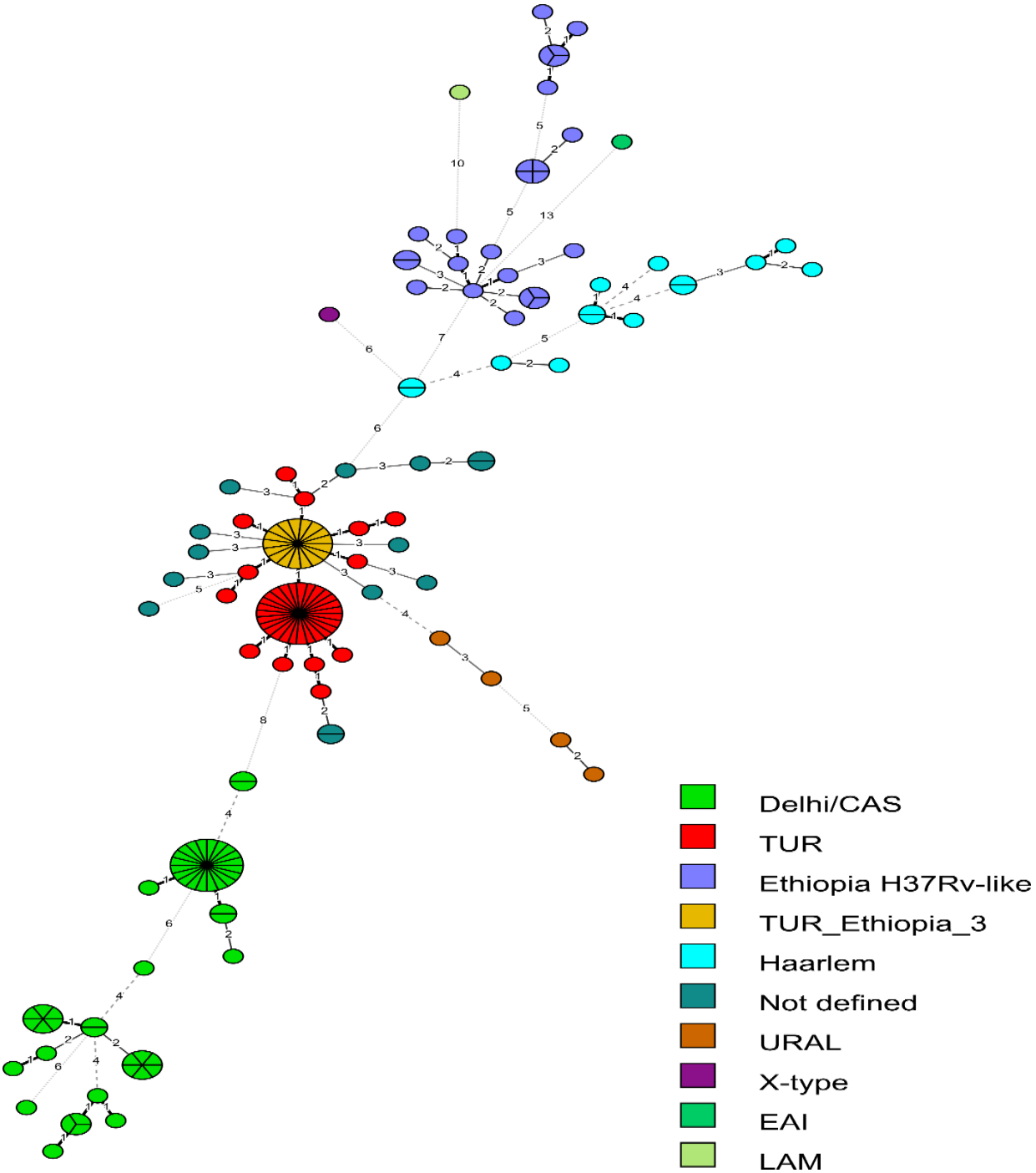


Figure 5-2: Minimum spanning tree based on the diversity of MIRU-VNTR 24-loci data. The different complexes identified are colored by the set of 24-loci among the 167 *M. tuberculosis* strains analyzed. The size of each circle is proportional to the number of MIRU-VNTR types belonging to a particular complex.

Distribution of drug target genes' mutations among lineages

Seventy-six (45.5%) isolates had the mutations in the rifampicin resistance determining region of rpoB gene (RRDR), and majority 26 (34.2%) of the mutations were found in Delhi/CAS and 17 (22.4%) were from TUR, predominantly replacing serine with leucine in 48 (96.0%) strains and serine substituting by tryptophan in 2 (4.0%) strains. Besides the second highest 14 (18.4%) proportion of mutation was observed in TUR, TUR_ Ethiopia _3 and EAI linages, at codon 526 (H526Y [CAC to TAC], H526S [CAC to AGC], H526D [CAC to GAC], and H526L [CAC → CTC]) and a majority amino acid change was observed at codon H526Y in 10 (13.2%) strains of TUR lineage.

Table 5-3: Drug resistance Pattern in M. tuberculosis complex isolates based on the genotyping January, 2017 (n=167)

Genotyping	INH Non- MDR-TB resistance (n=17) n (%)	STR Resistance (n=82), n (%)	EMB resistance (n=63), n (%)	PZA resistance (n=59), n (%)	INH and RIF (MDR-TB) (n=76), n (%)	All 5 drugs resistance (n=37), n (%)
Delhi/CAS (n=50)	4 (23.5)	29 (35.4)	25 (39.7)	24 (40.7)	27 (35.5)	17(45.9)
EAI (n=1)	0 (0.0)	1 (1.2)	1 (1.6)	1 (1.7)	1 (1.3)	1 (2.7)
H37Rv-like (n=25)	2 (11.8)	5 (6.1)	4 (6.3))	2 (3.4)	3 (3.9)	2 (5.4)
Haarlem (n=14)	1 (5.9)	2 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LAM (n=1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)	0 (0.0)
TUR (n=44)	6 (35.3)	34 (41.5)	25 (39.7)	25(42.4)	35 (46.1)	14 (37.8)
TUR_Ethiopia_3 (n=17)	3 (17.6)	8 (9.8)	4 (6.3)	4 (6.8)	6 (7.9)	0 (0.0)
Undefined (n=10)	1 (5.9)	2 (2.4)	2 (3.2)	2 (3.4)	2 (2.6)	2 (5.4)
URAL (n=4)	0 (0.0)	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)
X (n=1)	0 (0.0)	1(1.2)	1 (1.6)	1 (1.7)	1 (1.3)	1 (2.7)

As shown in table 5.4, 86 (51.5%) mutations were found in katG gene in lineage of TUR, Delhi/ CAS, TUR_ Ethiopia _3, H37Rv-like, undefined, EAI, Haarlem and X linages, and the highest prevalence was found in TUR with 40 (46.5%), followed by Delhi/CAS with 29 (33.7%) and 9 (10.5%) TUR_ Ethiopia _3 strains. The highest proportion of mutation was observed at codon 315

in 84 (97.7%), that predominantly replacing serine substituting by threonine (A**G**C→ACC) in nearly all isolates and the highest proportion was found in TUR with 39 (46.4%), followed by Delhi/CAS with 27 (32.1%) and TUR_ Ethiopia _3 9 (10.7%). Additionally, among INH resistance strains, there were mutations in fabg1-inhA at -15 and -8 by substitution of C to T, and T to C in 4 (66.7%) TUR family respectively

Moreover, 63 (37.7%) mutation was found in embB gene in TUR, Delhi/ CAS, TUR_ Ethiopia _3, H37Rv-like, undefined, EAI, and X linages, and the highest prevalence was found in TUR with 28 (44.4%), followed by Delhi/CAS with 25 (39.7%) strains. The highest proportion of mutation was observed at codon 306 in 41 (65.1%) among 4 lineages such as TUR, Delhi/ CAS, EAI, TUR_ Ethiopia _3, H37Rv-like, and undefined, and the highest proportion was Delhi/CAS with 21 (51.2%), followed by TUR with 14 (34.1%) strains, that predominantly replacing methionine with isoleucine (ATG → ATC, ATG → ATC) in 25 (52.1%) isolates, and methionine substituting by valine (ATG → GTA) and majority 21 (51.2%) of the isolates were from Delhi/CAS table 5.4.

A total of 61 (36.5%) isolates had a mutation in pncA gene; of these 25 (41.0%) were from TUR, and the second highest 24 (39.3%) proportion was from Delhi/CAS lineage. The highest rate of mutation was found between position 192–193 (codon 64 and 65) due to insertion of A in 16 (26.2%) Delhi/CAS strains, and the second highest rate mutation was identified from 5 (8.2%) strains of TUR at codon 130 (V130G [GTG → GGG] and V130M [GTG → ATG]). Moreover, as shown in table 5.4, 43 (25.8%) isolates had the mutations in rpsL and 9 (5.3%) had in rrs genes. A high 37 (86%) proportion of mutations in rpsL gene were found at codon 88 (K88R and K88T), and the highest prevalent mutants were from Delhi/CAS 12 (19.0%) and 10 (15.9%) TUR lineage. Furthermore, mutations in rrs gene were identified at position 514 (A → C), 517 (C→T), 613 (A → C), 891 (G → A), (906 A → G), and 1010 (A → C) with the highest prevalence of Delhi/CAS in 5 (55.6%), and followed by with TUR 3 (33.3%) strains.

Table 5-4: Drug target genes mutation of *M. tuberculosis* based on the genotyping January, 2017 (n=167)

Gene	Nucleotide change	Amino acid change	Lineage									
			Delhi/ CAS	EAI	H37Rv-like	Haarlem	TUR	TUR_ Ethiopia _3	Undefined	URAL	X	LAM
	GAC→GTC	D516V	-	-	-	-	1 (1.3)	3 (3.9)	-	-	-	-
	CAC→ GAC	H526D	-	-	-	-	1 (1.3)	-	-	-	-	-
rpoB	CAC→ CTC	H526L	-	1 (1.3)	-	-	-	-	-	-	-	-
(n=76)	CAC→AAC	H526S	-	-	-	-	-	2 (2.6)	-	-	-	-
	CAC→TAC	H526Y	-	-	-	-	10 (13.2)	-	-	-	-	-
	CTG→ CCG	L533P	-	-	-	-	2 (2.6)	-	-	-	-	-
	CTG→ GTG	L538V	1 (1.3)	-	-	-	-	-	-	-	-	-
	A inserted	Q513 insA	-	-	1 (1.3)	-	-	-	-	-	-	-
	CAA→ CCA	Q513P	-	-	-	-	3 (3.9)	-	-	-	-	-
	TCG→ TTG	S522L	-	-	-	-	1 (1.3)	-	-	-	-	-
	TCG→TTG	S531L	26 (34.2)	-	2 (2.6)	-	17 (22.4)	1 (1.3)	1 (1.3)	-	1 (1.3)	-
	TCG→TGG	S531W	-	-	-	-	1 (1.3)	-	1 (1.3)	-	-	-
	661-662 ins G	221fs	-	-	-	-	1 (1.2)	-	-	-	-	-
katG		E334S	1 (1.2)	-	-	-	-	-	-	-	-	-
(n=86)	AGC→AG**A**	S315H	1 (1.2)	-	-	-	-	-	-	-	-	-
	A**G**C→ACC	S315T	27 (31.4)	1 (1.2)	4 (4.7)	1 (1.2)	39 (45.3)	9 (10.5)	1 (1.2)	-	1 (1.2)	-
	T- → C	T-8C	-	-	-	-	1 (16.7)	-	-	-	-	-
fabG1 -inhA	C- →T	C-15T	-	-	-	-	3 (50.0)	-	-	-	-	-
(n=6)	G→C	G28C	-	-	-	-	-	-	-	-	1 (16.7)	-
	T→G	T14G	1 (16.7)	-	-	-	-	-	-	-	-	-
	AGT→TGT, 1204 del C	347fs S402C*	1 (1.6)	-	-	-	1 (1.6)	1 (1.6)	-	-	-	-
	1101-1102 ins G	368fs	1 (1.6)	-	-	-	-	-	-	-	-	-

	GCG→CCG	A409P	-	-	-	-	1 (1.6)	-	-	-	-	-
	GAT→ CAT,	D328H, D354A	-	-	-	-	1 (1.6)	-	-	-	-	-
embB (n=63)	GAG→GCG, GGC→GAC	E378A,G406D	-	1 (1.6)	-	-	-	-	-	-	-	-
	GGC→GCC	G406A	1 (1.6)	-	-	-	11 (15.9)	-	-	-	1 (1.6)	-
	ATG→ ATA	M306I*	6 (9.5)	-	1 (1.6)	-	11 (17.5)	1 (1.6)	1 (1.6)	-	-	-
	ATG→ ATA, 1039 del A	M3-6I, 347fs*	-	-	-	-	1 (1.6)	-	-	-	-	-
	ATG→ CTA	M306L	-	-	-	-	-	1 (1.6)	1 (1.6)	-	-	-
	ATG→ GTA	M306V	15 (23.8)	-	1 (1.6)	-	2 (3.2)	-	-	-	-	-
	ATG→AGG, GAT→ GCT	M316R, 354A*	1 (1.6)	-	-	-	-	-	-	-	-	-
	AGT→TGT, 1204 del C	S347C, 402fs*	-	-	-	-	-	1 (1.6)	-	-	-	-
	TGG→GGG	W332V	-	-	-	-	1 (1.6)	-	-	-	-	-
pncA (n=61)	A→ G	-11	-	-	-	-	1 (1.6)	-	1 (1.6)	-	-	-
	300-301 ins GC	100-101fs	-	-	1 (1.6)	-	-	-	-	-	-	-
	38--388 del AGGTCGATG	127fs	-	1 (1.6)	-	-	-	-	-	-	-	-
	529-530 ins A	177fs	-	-	-	-	1 (1.6)	-	-	-	-	-
	GCA→GTA	A102V	-	-	-	-	4 (6.6)	-	-	-	-	-
	TGT→TGG	C138W	-	-	-	-	1 (1.6)	-	-	-	-	-
	TGC →CGC	C72R	-	-	-	-	-	1 (1.6)	-	-	-	-
	GAT→ AAT	D129N	-	-	-	-	1 (1.6)	-	-	-	-	-
	GAC→ GCC	D12A	-	-	-	-	1 (1.6)	-	-	-	-	-
	GGA→GCC	G108A	-	-	-	-	-	1 (1.6)	-	-	-	-
	GGA→GAA 547 del G	G108E, 183fs*	-	-	-	-	-	-	1 (1.6)	-	-	-
	GGC→AGC	G78S	-	-	-	-	-	1 (1.6)	-	-	-	-
	CAC→TAC	H57Y	1 (1.6)	-	-	-	-	-	-	-	-	-
	AAG→GAG	K48E	-	-	-	-	1 (1.6)	-	-	-	-	-

	TTG→ TGG	L182W	-	-	1 (1.6)	-	-	1 (1.6)	-	-	-	-
	192-193 Ins A	S65fs	16 (26.2)	-	-	-	-	-	-	-	-	-
	ACT→ CCT	T76P	-	-	-	-	3 (4.9)	-	-	-	1 (1.6)	-
	GTG→ GGG	V130G	-	-	-	-	5 (8.2)	-	-	-	-	-
	GTG→ATG	V130M	-	-	-	-	2 (3.2)	-	-	-	-	-
	GTG→GCG	V139A	4 (6.6)	-	-	-	-	-	-	-	-	-
	GTG→ GCG	V155A	1 (1.6)	-	-	-	-	-	-	-	-	-
	GTC → TTC	V180F	-	-	-	-	1 (1.6)	-	-	-	-	-
	GTC → GGC	V44G	-	-	-	-	1 (1.6)	-	-	-	-	-
	GTC→ GGC	V7G	-	-	-	-	1 (1.6)	-	-	-	-	-
	TGG→CGG	W119R	1 (1.6)	-	-	-	-	-	-	-	-	-
	TGG→TGA	W119STOP	-	-	-	-	1 (1.6)	-	-	-	-	-
	TAC→ CAC	Y1-3H	-	-	-	-	2 (3.2)	-	-	-	-	-
		Y41	-	-	-	-	1 (1.6)	-	-	-	-	-
	TAC→TAG	Y41STOP	-	-	-	-	1 (1.6)	-	-	-	-	-
rpsL	AAG→AGG	K43R	5 (11.6)	-	-	-	1 (2.3)	-	-	-	-	-
(n=43)	AAG→AGG	K88R	10 (23.3)	-	1 (2.3)	1 (2.3)	5 (11.6)	1 (2.3)	-	1 (2.3)	-	-
	AAG→ACG	K88T**	2 (4)	1 (2.3)	1 (2.3)	-	1 (27.9)	1 (2.3)	-	-	1 (2.3)	-
rrs	A → G	1-1-a>c¥	-	-	-	-	-	-	1 (11.1)	-	-	-
(n=9)	A → C	514a>C¥	1 (11.1)	-	-	-	1 (11.1)	-	-	-	-	-
	C → T	517c>T¥	1 (11.1)	-	-	-	1 (11.1)	-	-	-	-	-
	A → C**	631a>c *,¥	1 (11.1)	-	-	-	-	-	-	-	-	-
	G → A*	891g>A¥	1 (11.1)	-	-	-	-	-	-	-	-	-
	A → G	906a>G¥	1 (11.1)	-	-	-	1 (11.1)	-	-	-	-	-

* Gene that has multiple mutations, ** Multiple mutations was found in rpSL (K88T) and rrs (631, A → C) , ¥ numbers indicated nucleotide position' fs: frameshift, del: deletion, ins: insertion

Population structure and cluster analysis

Cluster analysis showed that a total of 103 (61.7%) strains shared a genotyping pattern with at least one other isolate, and they were grouped in 19 clusters ranging in size from 2 to 26 strains; the remaining 64 (28.3%) strains were categorized into unique genotypes. Moreover, a recent transmission index (RTI) was determined and found to be 50.3%. Besides, strains were also assigned to multiple locus VNTR analysis (MLVA) MtbC15-9 types, the largest cluster (n = 26; cluster 4: MLVA MtbC15-9 type 3554-15) is formed by the TUR genotype followed by the second largest clusters formed by strains of the Dehli/CAS genotype (n = 21, cluster 19: MLVA MtbC15-9 type 1064-32). Interestingly all TUR_Ethiopia-3 genotype was clustered in one group, (n = 17; cluster 5: MLVA MtbC15-9 type 594-15). These findings indicate that there is an ongoing transmission of these strains (Figure 5.1, Annex V).

Moreover, of 76 MDR-TB strains, 58 (76.3%) strains were grouped in 8 clusters ranging in size from 2 to 24 strains,. The highest clusters that had 24 MDR strains were TUR (MLVA MtbC15-9 type 3554-15), and followed by Dehli/CAS (MLVA MtbC15-9 type 1064-32) genotype contained 17 strains, and the third largest cluster formed by TUR_Ethiopia_3 (MLVA MtbC15-9 type 594-15) genotype contained 5 strains. In addition, a recent transmission index (RTI) finding among MDR-TB was 65.8%, indicating a transmission of MDR strains in the population.

Table 5-5: Discriminatory capacities of spoligotyping, MIRU-VNTR 24-loci, and combination of spoligotyping and MIRU-VNTR 24-loci for *M. tuberculosis* isolates from Ethiopia, January, 2017 (n=167)

Genotyping method	No. of different patterns	No. of isolates with unique pattern	No. of Clusters	No. of isolates in clusters	Clustering rate (%)	RTI (%)
Spoligotyping	37	26	11	141	84.4	77.8
MIRU-VNTR 24-loci	73	54	19	113	67.7	56.3
MIRU-VNTR 24-loci and Spoligotyping	82	64	19	103	61.7	50.3

Of 58 clustered MDR-TB strains, 39 (67.2%) strains (16 Dehli/CAS and 13 TUR genotypes) had a mutation at codon S531L and S315T of rpoB and katG genes respectively. Moreover, the

second high proportion of mutation was observed in rpoB at codon H526Y/D/S with 10 (17.2%) strains (8 TUR and 1 TUR_Ethiopia_3 genotypes) and all had also mutation at codon S315Tof katG gene. In another finding, 35 (62.5%) strains had the mutation in the embB gene and about one third, 21 (60%) showed mutation at codon M306V/I. In addition to this, 6 clusters contained 29 MDR strains total were resistance to EMB, PZA, and STM. Moreover, out of 103 clustered strains, 73 (70.9%) were resistance to any drugs, such as 8 clusters contained 49 EMB resistance, 9 clusters contained 48 PZA resistance, 9 clusters contained 67 INH resistance, and 10 clusters contained 57 STR resistance. These clustering are indicating transmission of resistant strains

Factors associated with strain clustering

When the clustering rates were stratified for strains of different phylogenetic lineages, interestingly all TUR_Ethiopia_3 lineage were clustered in one group. When we analyzed the odd, the odds of clustering was more than 5-fold higher among Dehli/CAS lineage (39 out of 50 strains) (P=0.000), nearly 4-fold higher among TUR lineage (31 out of 44 strains) (P<0.015), compared to H37Rv like lineage [10 out of 25 strains] (Table 5.6). The odds of clustering was also nearly 5-fold higher among PZA resistant strains (49 out of 59 strains) compared to PZA susceptible strains (P=0.000), more than 3-fold higher among RIF resistant strains (58 out of 76 strains) compared to RIF susceptible strains (P=0.000), 3-fold higher among INH resistant strains (68 out of 93 strains) (P=0.001) compared to INH susceptible strains, and nearly 4-fold higher among EMB resistant strains (50 out of 63 strains) (P=0.001) compared to EMB susceptible strains. Furthermore, STR resistance was a significant risk factor for clustering (58 out of 82 strains) (OR=2.148, P = 0.019) compared to STR susceptible strains.

In another finding bivariate analysis indicated that multidrug resistance was a significant risk factor for clustering of 58 out of 76 strains (OR=3.29, P = 0.000) compared to non-multidrug-resistant strains. Moreover, age (p=0.735), sex (p=0.456), marital status (p=0.720), HIV stauts (p=0.179), TB treatment hostroy (p=0.185), family member who had previously TB infected cases (p=0.183),

antibiotic taking (p=0.630), alcohol drinking (p=0.362), cigarette smoking (p=0.329), health facility visit (p=0.710) and hospital admission (p=0.440), were not a significant risk factor for clustering (Table 5.6).

Table 5-6: Demographic characteristics of the study subjects, drug resistance patterns, phylogenetic lineages and their association with clustering

Variable	Total (n=167)	Genotyping patterns		Odds ratio (95% CI)	P-Value
		Clustered	Unique		
Sex					
Male	101	60 (59.4)	41 (40.6)	1.278 (0.67 2.43)	0.456
Female	66	43 (65.2)	23 (34.8)	1	
Age group					
15-24	21	13 (61.9)	8 (38.1)	1	
25-34	73	46 (63.0)	27 (37.0)	1.048 (0..39 2.85)	0.735
35-44	52	30 (57.7)	22 (42.3)	0.839 (0.29 2.37)	
45-54	12	9 (75.0)	3 (25.0)	1.846 (0.59 10.29)	
55 ≥	9	5 (55.6)	4 (44.4)	1.625 (0.44 13.75)	
Marital Status					
Divorced/ Widow	8	4 (50)	4 (50.0)	0.769 (0.39 1.51)	0.441
Married	100	60 (60.0)	40 (40.0)	0.857 (0.38 1.94)	0.710
Unmarried	59	39 (66.1)	20 (33.9)	1	
Residence					
Urban	155	97 (62.6)	58 (37.4)	0.926 (0.31 2.74)	0.889
Rural	12	6 (50.0)	6 (50.0)	1	
Cigarettes smoking					
Yes	21	15 (71.4)	6 (28.6)	1.648 (0.60 4.49)	0.329
No	146	88 (60.3)	58 (39.7)	1	
Alcohol drinking					
Yes	32	22 (68.8)	10 (31.2)	1.467 (0.64 3.34)	0.362
No	135	81 (60.0)	54 (40)	1	
HIV status					
Positive	97	64 (66.0)	33 (34.0)	1.542 (0.82 2.90)	0.179
Negative	70	39 (55.7)	31 (44.3)	1	
Antibiotic treatment history					
Yes	64	37 (57.8)	27 (42.2)	0.854 (0.45 1.62)	0.630
No	103	66 (64.1)	37 (35.9)	1	
TB treatment history					
Retreatment	112	74 (66.1)	38 (33.9)	1.75 (0.91 3.37)	0.097
New	55	29 (52.7)	26 (47.3)	1	
Previously TB infected Family member					
Yes	65	36 (55.4)	29 (44.6)	0.648 (0.34 1.23)	0.183
No	102	67 (65.7)	35 (34.3)	1	
Health facility visit					
Yes	138	86 (62.3)	52 (37.7)	1.167 (0.52 2.64)	0.710
No	29	17 (58.6)	12 (41.4)	1	
Hospital admitted					
Yes	39	22 (56.4)	17 (43.6)	0.751 (0.36 1.56)	0.440
No	128	81 (63.3)	47 (36.7)	1	
Ethambutol					
R	63	50 (79.4)	13 (20.6)	3.701 (1.80 7.61)	0.000
S	104	53 (51.0)	51 (49.0)	1	
Isoniazid					
R	93	68 (73.1)	25 (26.9)	3.031 (1.59 5.79)	0.001
S	74	35 (47.3)	39 (52.7)	1	

Pyrazinamide					
R	59	49 (83.1)	10 (16.9)	4.900 (2.51 10.67)	0.000
S	108	54 (50.0)	54 (50.0)	1	
Rifampicin					
R	76	58 (76.3)	18 (23.7)	3.29 (1.69 6.44)	0.000
S	91	46 (50.5)	45 (49.5)	1	
Streptomycin					
R	82	58 (70.7)	24 (29.3)	2.148 (1.13 4.10)	0.019
S	85	45 (52.9)	40 (47.1	1	
MDR-TB					
R	76	58 (76.3)	18 (23.7)	3.29 (1.69 6.44)	0.000
S	91	46 (50.5)	45 (49.5)	1	
***M. tuberculosis* lineages**					
Delhi/CAS	50	39 (78.0)	11 (22.0)	5.318 (1.87 15.1)	0.000
Haarlem	14	4 (28.6)	10 (71.4)	0.600 (0.15 2.50)	0.792
TUR	44	31 (70.5)	13 (29.5)	3.577 (1.28 10.0)	0.015
TUR_Ethiopia_3	17	17 (100)	0 (0.0)	-	-
Undefined	10	8 (80.0)	2 (20.0)	0.375(0.07 0.15)	0.70
H37RV-Like	25	10 (40.0)	15 (60.0)	1	
URAL	4	0 (0.0)	4 (100.0)	-	-
EAI	1	0 (0.0)	1 (100.0)	-	-
LAM	1	0 (0.0)	1 (100.0)	-	-
X	1	0 (0.0)	1 (100.0)	-	-

Remarkably, about half, 35 (46.1) MDR-TB strains were classified as TUR lineage. The odds of TUR strain having multidrug resistance was 7-fold higher (P =0.000) compared to patients with the non-TUR strains, in addition to this, the odds of a TUR strain having INH resistance was 19-fold higher (P =0.000) compared to patients with the non-TUR strains. Similarly, the third high odds of resistance to STR-drugs was found to be 5-fold higher among patients with a TUR strain (P=0.000) compared to patients with non-TUR strains. Moreover, a significantly higher risk of resistance to EMB (P=0.017), and PZA (P<0.001), was observed among patients with a TUR lineage compared to patients with the non-TUR strains (Table 5.7). However, odds of resistance to all first-line anti-TB-drugs was not a significantly associated with among patients with a TUR strain (P=0.075) compared to patients with non-TUR strains.

Table 5-7: *M. tuberculosis* TUR lineage and its association with anti-TBdrug resistance

Drug resistance		*M. tuberculosis* lineages		OR (95% CI)	P-value
		TUR n (%)	Non-TUR n (%)		
Ethambutol					
	R	25 (69.4)	11 (30.6)	2.94 (1.455.98)	0.003
	S	19 (14.5)	112 (85.5)	1	
Isoniazid					
	R	41 (44.1)	52 (55.9)	18.66 (5.48 63.56)	0.000
	S	3 (4.1)	71 (95.9)	1	
Pyrazinamide					
	R	25 (42.4)	34 (57.6)	3.44 (1.68 7.04)	0.001
	S	19 (17.6)	89 (82.4)	1	
Rifampicin					
	R	35 (46.1)	41 (53.9)	7.78 (3.42 17.71)	0.000
	S	9 (9.9)	82 (90.1)	1	
Streptomycin					
	R	34 (41.5)	48 (58.5)	5.31 (2.41 11.74)	0.000
	S	10 (11.8)	75 (88.2)	1	
MDR-TB					
	R	35 (46.1)	41 (53.9)	7.78 (3.42 17.71)	0.000
	S	9 (9.9)	82 (90.1)	1	
Resistant to all 5 drugs					
	R	14 (37.8)	23 (62.2)	2.03 (0.93 4.43)	0.075
	S	30 (23.1)	100 (76.9)	1	

5.5 Discussion

In this study, the genotypic diversity and drug resistance of *M. tuberculosis* strains in Ethiopia were characterized. According to the phylogenetic classification of *M. tuberculosis* isolates using both the MIRU-VNTR 24- loci profiles and spoligotyping patterns, a total of 167 strains were classified into ten groups such as Delhi/CAS, TUR, H37Rv-like, TUR_ Ethiopia _3, Haarlem, EAI, LAM, undefined, URAL, and X It was observed that the genetic diversity of *M. tuberculosis* isolates in this study was similar compared to the previous studies done in Ethiopia (Fantahun *et al.,* 2015, Tessema *et al.,* 2013 Agnoafir *et al.,* 2010). Moreover, in our study, Dehli/CAS was the predominant lineage in Ethiopia, accounting for 30% of the identified *M. tuberculosis* strains. It was comparable with several studies reported from Ethiopia (Garedew *et al.,* 2013, Mulugeta M, *et al.,* 2014, Amare *et al.,* 2012, Diriba *et al.,* 2013, Adane *et al.,* 2012, Biadglegn *et al.,* 2015). Similarly, several studies revealed that the proportion of the Delhi/CAS lineage was ranging from 12–68% (Mulugeta *et al.,* 2014, Al-Hajoj *et al.,* 2013, Diriba *et al.,* 2013, Adane *et al.,* 2012).

Moreover, other studies from neighbor countries also showed that *M. tuberculosis* Dehli/CAS is the predominant lineage in Sudan 49% (Eldin *et al.,* 2011) and Kenya 28.9% (Ndungu *et al.,* 2017) and Djibouti 21.9% (Godreuil *et al.,* 2010) of investigated strains. In addition to this, Delhi/CAS has also been identified as predominant lineage in Saudi Arabia 26.4% (Al-Hajoj 2010), Pakistan 61% (Tanveer *et al.,* 2008) and Iran (Jafarian *et al.,* 2010). As Dehli/CAS lineage is predominant lineage in Central Asia such as India (Bhanu *et al.,* 2002) and Middle-East, there are assumptions that would explain the presence of the Dehli/CAS lineages in Ethiopia: (i) it could have emerged in Ethiopia and migrated to the Middle East and Central Asia, a hypothesis in agreement with the suggestion that East Africa is the cradle of *M. tuberculosis* complex species (Gutierrez *et al.,* 2005); this is supported by a recent evidence (Comas et. al 2013) that MTBC coevolved with the modern human host and migrated from East Africa to Asia and other parts of the world; (ii) alternatively, it could be due to migration of this lineage from the Middle East and Central Asia to Ethiopia due to the recent human migrations from these areas to Ethiopia as suggested by a previous study (Tessema *et al.,* 2013) and this might be indicating the successful transmitting of Delhi/CAS strains among the community due to extensive social relations and high mobility that could possibly expose them to greater risk of acquiring TB (Al-Hajoj *et al.,* 2013, Biadglegn *et al.,* 2015).

In another finding, the second highest prevalent was TUR with 26.3%, this is higher than previously reported from Ethiopia by Tessema *et al.,* (2013) 2.1%, and Biadglegn *et al.,* (2015) 0.5%. Interestingly, several studies done in Ethiopia on genotyping analysis did not report any TUR lineage. This might be indicating the presence of active transmission in the population, or as TUR lineage was chronologically originated in Turkey. It is hypothesized that the strain was introduced in Ethiopia because of the large Turkey community in Ethiopia due to the growing economic partnerships between Ethiopia and Turkey governments, so it may have contributed in the introduction of this lineage and might be indicating the active transmission of TUR strains in

the community. This assumption also supports by Al-Hajoj *et al.,* (2013) and Biadglegn *et al.,* (2015).

Surprisingly, drug resistance analysis also revealed that more than 46% of MDR-TB strains were observed in TUR lineage, and about 36% strains were from Delhi/CAS, and TUR_Ethiopia_3 (8%), and the remaining proportion (10%) was belonging to H37Rv-like, X, and EAI lineages. These results seem to argue in favor of both TUR and Delhi/CAS, and inadequate monitoring and control of drug-resistant TB. Moreover, the pattern of multidrug resistance within the lineage was found to be higher among TUR with 79.5%, and followed by Delhi/CAS with 54.0%. The finding indicates the high prevalence of TUR and Delhi/CAS in MDR-TB. This may be due to the fact that as Addis Ababa is the capital city and one of the diplomatic city in the world, it has a mixed population from a different part of the world, especially the current Ethio-Turkish, India and China Investment relationship would contribute for these. In addition to this, as RTI finding was high (50.3%), this clearly indicates an active transmission of TUR strains in the community. In another hand, no MDR-TB strain was identified from Haarlem lineage, this is inconsistent with the studies done in Ethiopia by Tessema *et al.,* (2013) and Mulalem (Mulalem *et al.,* 2010). The studies done in Northwest Ethiopia and Tunisia showed that the Haarlem family genotype had a significant association between infection with multi-drug resistance, and rapid clonal expansion (Tessema *et al.,* 2013, Mardassi *et al.,* 2005). The possible reasons for the difference could be due to the fact that this study was conducted among population presumptive MDR-TB patients, and the Haarlem lineage circulating in the study area might be wild-type strain that different from circulating in Northwest Ethiopia and Tunisia.

Furthermore, TUR clade is followed by H37Rv like (15.0%), TUR_Ethiopia (10.2%), Haarlem (8.4%), Unidentified (6.0%), Ural (2.4%), and LAM, X-type, and EIA with 0.6% each (Table 5.3). This observation emphasizes the complex diversity of circulating *M. tuberculosis* strains in Ethiopia that could reflect the different transmission pathways occurring within the country.

Besides, it has been suggested that particular lineages of *M. tuberculosis* might be adapted to specific human population (Helal *et al.,* 2009). A large number of patterns shows a large diversity in our study. This diversity could be attributed to human travel and migration because of the political instability in the neighboring countries such as Somalia and South Sudan which has resulted in an influx of refugees from these countries. So movements of people may have played a major role in the transmission of the strains type among those countries. Moreover, this may be due to the fact that Addis Ababa, as a city of Africa Union, has a mixed population from a different part of the continent and globally, so that it could contribute for a large diversity of the lineages.

Furthermore, while it is well known that the drug-resistant TB is a result unsuccessful of TB program due to chromosomal alterations because of mutations or deletions, there are several factors related to TB control program that has a significant impact on the increasing and transmission of drug-resistant TB (Migliori *et al.,* 2010). Our study revealed about 80% TUR strains were MDR-TB it is very significant figure compared to other lineages. Bivariate analysis revealed that being TUR strain had a statistically significant association with developing of drug resistance to RIF (p=0.000), INH (p=0.000), STR (p=0.000), EMB (P=0.017), and PZA (P<0.001) drugs. All of these factors would appear to be predictors for TUR strain for development of multidrug resistance than other lineages. The environment might favor the survival and transmission advantage of this lineage, and being a TUR could be a predisposing factor for the drug target gene mutation. In another finding of the study, 54% the Delhi/CAS lineage were multi-drug resistance strains and had addition resistance to STR, EMB, and PZA. This finding was comparable with the previous study from India (Stavrum *et al.,* 2009). Similarly, the environment might favor the survival and transmission including drug target gene mutation advantage like TUR. However, it is very important to understand the fact that whether specific lineages are overrepresented among drug-resistant or successfully transmitted within the community.

More than 45% isolates had the mutations in the rifampicin resistance determining region of rpoB gene, and about 66% of the mutation occurred at codon 531 of the rpoB gene, and more than one-third (34.2%) of the mutation was found in Delhi/CAS and with approximately one-fourth (22.4%) in TUR. Moreover, the second highest prevalent nucleotide changes were observed in 18.4% isolates at codon 526, about two-thirds strains had a mutation substituting of histidine to tyrosine, and all these mutants were from TUR. As such, differences in the frequencies of mutations in the 526 and 531 codons of the rpoB gene among isolates of the Delhi/CAS and TUR. But a study done in Saudi Arabia showed that the comparable number of Delhi/CAS strains had a mutation at codon 531 (Varghese *et al.,* 2014). However, no TUR family reported as a resistant RIF in that study. The difference could be due to the fact that the frequencies and types of mutations in one country or geographical region may not be generally applicable. In supporting our study, a multicenter study revealed that significant geographic differences in the types of polymorphisms and frequencies were observed (Hoshide *et al.,* 2013).

Furthermore, we found a mutation in katG gene in 51.5% of the isolates, and almost all isolates had a mutation at codon 315, and nearly all mutations occurred in serine substituting by threonine, and the highest proportion was found in TUR (46.4%) and Delhi/CAS (32.1%) lineages. A study done in Iran showed that about 16% of the Delhi/CAS had a mutation at codon 315, (Doustdar *et al.,* 2008). Further, other studies showed that a high frequency of the *kat*G S315T mutation in INH resistant *M. tuberculosis* isolates of the Haarlem strain family was reported from South Africa (Dalla *et al.,* 2009) and Tunisia (Mardassi *et al.,* 2005). These difference might be also due to the geographic differences. A study done by Hoshide *et al.,* (2013) showed that a significant geographic difference was served in the types of mutations and frequencies. Additionally, among INH resistance strains, there were mutations in -15 and -8 by substitution of C to T, and T to C in TUR family respectively. It is comparable to the study done by Varghese *et al.,* (Varghese *et al.,* 2014).

In addition, more than one-third (37%) the isolates had a mutation in the embB gene and the highest proportion of mutation was observed at codon 306 in 65.1% of the isolates, that predominantly replacing methionine with isoleucine and valine, and more than 50% of the isolates were from Delhi/CAS lineage. This major mutation at codon 306 was predominately found in MDR-TB strains, our study suggests that there might be an association between the embB codon 306 mutation and katG and rpoB gene. Another study also revealed that embB codon306 mutations were associated with resistance to anti-TB drugs, which may be a predisposed factor for the development of resistance to anti-TB drugs (Safi *et al.,* 2008). The findings from our and previous study help us to conclude that this mutation might be favor both TUR and Delhi/CAS lineages for survival and transmission in the population, although further study is important.

Among pncA gene mutants; the highest proportion was from TUR (41%) and Delhi/CAS (39.3%) lineage. Mutations in pncA gene were highly diverse and distributed throughout the gene were observed in more than 25 codons that causing amino acid substitutions. This finding is similar to previous studies (Chang *et al.,* 2011; Cui *et al.,* 2013). The highest mutation was found between codons 64 and 65 pncA gene by insertion of nucleotide A at 26.2% strains (all are Delhi/CAS family). Furthermore, about 26% isolates had the mutations in rpsL gene at codon 88 (86%) and the highest proportion was from Delhi/CAS (48%) and 10 (40%) TUR lineage. This is inconsistent with the study of (Lipin *et al.,* 2007) who found that the high prevalent mutation was found at codon 43 in 27% of Haarlem family, and 75% of Beijing family isolates. However in our study, out of six, mutants 5 strains were from Delhi/CAS. The possible explanation would be due to the fact that the since different strains are circulating in a different region, frequencies and types of mutations also can vary from region to region.

Even though the interpretations of clustering results remain somewhat controversial, it is accepted that clustering is a marker for recent transmission and using recent TB transmission rate index, it can be estimated the efficacy of the TB control program (Niemann *et al.,* 2000, Tessema *et al.,*

2013). So the high TB incidence and the prevalence drug-resistance rates in Ethiopia are indicators for a weakness of the TB control program (Tessema *et al.,* 2013), in supporting this assumption, our study found a high rate of clustering with 61.7% of the total strains in the study. This is higher than the previous reports from the Addis Ababa and Northwest Ethiopia that showed clustering rate of 41.2% (Bruchfeld *et al.,* 2002) and 45.1% (Tessema *et al.,* 2013) respectively. This high clustering rate indicates that the recent and ongoing transmission of the drug-resistant strains in the population is increased, and it also shows a weakness of the TB control program.

Likewise, there was a significant association between recent transmission and infection with INH, RIF, PZA, EMB resistant strains, and patients with MDR strains. It was comparable to the study done by Tessema *et al.,* (2013) in Ethiopia. It could be due to the fact that, there is limited diagnostic TB laboratories for diagnosis of drug resistance in the country, so, drug resistant-TB is diagnosed after prolonged treatment with first-line anti-TB drugs. MDR-TB patient treatment with standard first-line drugs, instead of a regimen designed according to the resistance pattern has several potential adverse consequences such as patients remain on inadequate treatment longer, increasing the risk of treatment failure resulting death; selection of drug-resistant strains and patients remain infectious, increasing transmission to close contacts as well as community (Dorman and Chaisson 2007).

Remarkably, the finding revealed that there was a significant association between recent transmission and the TUR, Dehli/CAS, and TUR_Ethiopia_3 strain infections. Likewise, Gagneux *et al.,* proposed that the major *M. tuberculosis* lineages evolved so as to become adapted to specific host genetic backgrounds and are much more likely to transmit and cause disease among patients of the community (Gagneux *et al.,* 2006). These findings indicate a successful transmission of drug-resistant and MDR strains in the in the population, a situation that needs to be carefully monitored the trend of MDR-TB strains transmission to avoid catastrophe (Victor *et*

al., 2007). A comparison of our study findings with the other studies previously conducted in Ethiopia, there are major difference between our study and the previous studies that we found the highest prevalence of TUR and CAS families among MDR-TB strains so we suggest conducting further study in the country to well understand the genotyping circulation in the country for better control and prevention of MDR-TB.

5.6 Conclusion

In conclusion, our study showed that in addition to a highly diverse population structure of *M. tuberculosis* and high prevalence of drug-resistance, the high clustering and recent transmission rates observed underline active transmission of drug-resistant strains. The overall predominance strain was the Dehli/CAS lineage (29.9%) and ranked as the second predominant among MDR-TB strains with about 36%. Moreover, the highest predominant genotype among the MDR-TB strains was TUR lineage with more than 46%. There was a statistically significant association between TUR strain infection and multidrug resistance including the other first-line anti-TB drugs, (p=0.000). The high rate of recent transmission underlines active transmission of *M. tuberculosis* including drug-resistant strains, and as a result of a weak health system of TB control program in the Country. This emphasizes the importance of strengthening the health system in order to improve the TB/MDR-TB control and prevention program at large by supporting and improvement of TB diagnosis system including drug susceptibility test, and TB treatment and monitoring system. Further study should be conducted to insight the overall genotyping and drug resistance mechanisms in *M. tuberculosis* in the country.

Chapter 6: Publication IV

6. Evaluation of GeneXpert MTB/RIF® Molecular Assay using urine specimen for diagnosis of active pulmonary tuberculosis from sputum scarce patients in Ethiopia

Published at Journal of Diagnostic Techniques and Biomedical Analysis (2018 Vol: 7 Issue: 1)

6.1 Abstract

Tuberculosis (TB) is one of the leading causes of death in low-income countries especially among TB/HIV co-infected patients. Due to the weak health care system and difficulty of getting sputum specimens from TB-HIV co-infected patients and children, the rate of undiagnosed TB cases is very high in most high TB burden countries. Therefore, we evaluated the performance of GeneXpert MTB/RIF using urine as an alternative specimen for the sputum scarce patients.

Methodology: A total of 150 clinically TB suspected cases (75 sputum scarce cases and 75 cases who were capable to provide sputum) provided a morning urine specimen and 75 cases who were able to provide sputum also provided sputum. Urine and sputum specimens were analyzed by using GeneXpert MTB/RIF, culture and Ziehl-Neelsen (ZN) microscopy methods.

Result: Of the 150 urine and 75 sputum specimens tested, we found 5 (3.3%) urine and 26 (34.6%) sputum specimens positive by GeneXpert MTB/RIF and culture. Moreover, we found 1 (0.7%) urine and 1 (1.3%) sputum positive by GeneXpert MTB/RIF only. The sensitivity and specificity of GeneXpert MTB/RIF using urine were 100% and 99.3% (95%CI: 97.4-100%) respectively, in addition, using sputum specimens it had 100% sensitivity and 98.0% (95% CI: 94.8-100%) specificity by using culture results as reference test. The overall performance agreement between GeneXpert MTB/RIF and culture using urine and sputum specimens were 99.3% (95% CI: 97.4-100%) and 98.7% (95% CI: 96.1-100%) respectively. In general, our finding showed that the total TB confirmed cases were increased from 26 (17.3%) to 30 (20.0%) by using urine as an alternative specimen.

Conclusion: A good sensitivity and specificity of GeneXpert MTB/RIF were observed using urine specimen, thus, urine can be used as an alternative specimen for diagnosis of PTB from sputum scare patients using GeneXpert MTB/RIF Assay.

Key words: GeneXpert MTB/RIF. Tuberculosis, Urine, Sensitivity, Specificity.

6.2 Introduction

TB is one of the top ten causes of death worldwide and responsible for more deaths than Human Immuno-Deficiency Virus (HIV). It causes a disease among millions of people each year and it is the leading cause of death from an infectious disease worldwide (WHO, 2016). In 2016 there were about 10.4 million new TB cases, and 10% of the cases were HIV positive and the highest proportion was found in the WHO African Region. There were about 1.7 a million TB deaths, of these, about 0.4 million deaths resulting from HIV positive patients and the highest mortality rate was in the WHO African Region. Ethiopia is one of the 30th high burden countries (TB, TB/HIV and multi-drug-resistant TB [MDR-TB]), and TB remains one of the leading causes of mortality in the country. According to 2017 WHO report, 8% of the new TB cases were HIV positive, moreover, as one of the high TB/HIV and (MDR TB) burden countries, the prevalence MDR TB among previously treated and new TB cases was 14% and 2.7% respectively (WHO, 2016).

TB and HIV co-infections are associated with special diagnostic and therapeutic challenges and constitute an immense burden on healthcare systems of heavily infected countries like Ethiopia (FMOH, 2009). Ziehl–Neelsen (ZN) microscopy, the most accessible method in low and middle income countries for the diagnosis of PTB has low sensitivity, 20–70%. Up to 50% of patients with PTB are smear-negative and up to 10% of these patients remain culture-negative (Behr *et al.,* 1999) and the situation is worse in sputum-scarce TB patients (Gopinath *et al.,* 2007). Studies also showed that most of the smear and culture negative patients will develop TB disease in the course of time (Behr *et al.,* 1999, Dutt *et al.,* 1994).

In sputum-scarce PTB cases, bronchoalveolar lavage fluid and gastric lavage are a preferred clinical specimen in adults and children, respectively, but, bronchoalveolar and gastric lavage cannot be obtained with simple procedure and only found in well-equipped healthcare settings and have very low detection rates (Behr *et al.,* 1999, Dickson *et al.,* 2003). Several studies indicated that the detection rate of TB is improved by using urine specimen and they suggest that

urine could be a better alternative diagnostic specimen in sputum-scarce TB suspected cases (Torrea *et al.,* 2005, Gopinath *et al.,* 2007, Peter *et al.,* 2012). As Colby postulated, TB bacilli could be excreted through the kidneys and it could be found in the urine of TB patients who have no symptoms pertaining to the urinary tract (Colby 1961) and the null hypothesis confirmed later by studies done in HIV-negative (Kafwabulula *et al.,* 2002) as well as HIV-positive patients (Aceti *et al.,* 1999).

HIV-infected people have an annual risk of up to 15% of developing active TB once infected (Rieder 1999). More than 60 % of new cases of PTB patients are co-infected with HIV and found in developing countries (Corbett *et al.,* 2003). TB-related mortality is highest among HIV infected patients, and with HIV-related immunosuppression, disseminated forms of TB disease are very high compared to immuno-competent (Reid and Shah 2009) and diagnosing tuberculosis in HIV-infected patients is a major challenge since clinical and radiological findings are often atypical (Lawn and Wood 2011), and much more smear-negative pulmonary TB patients are not diagnosed surely (Davis *et al.,* 2010). Moreover, HIV patients are unable to produce sputum for diagnostic testing (Peter *et al.,* 2012). Diagnosis is therefore challenging and often delayed, and post-mortem studies reveal a large burden of undiagnosed TB in HIV-infected hospitalized patients (Cox *et al.,* 2010). Recent studies have indicated that the rapid initiation of anti-TB treatment may reduce mortality (Holtz *et al.,* 2011).

However, getting appropriate clinical specimen remains a major hindrance in HIV-infected and sputum scarce patients suspected of having active TB. There is a clear need for new, accurate, and rapid TB diagnostics, in 2010 WHO introduced molecular based GeneXpert/ MTB/RIF system for diagnosis of TB and Rifampin resistance TB from sputum and body fluid specimens (WHO, 2013b). There are studies done on evaluating the performance of the GeneXpert MTB/RIF molecular method using urine from sputum scarce patients, however, there is no published information in Ethiopia in sputum scarce patients using urine. Therefore, we hypothesized that

urine MTB/RIF may offer diagnostic utility in patients where a sputum-based diagnosis is not feasible. The objective of this study was to evaluate the performance of GeneXpert MTB/RIF for diagnosis of PTB using urine specimen as an alternative specimen from sputum scarce patients.

6.3 Materials and Methods

Study Setting and Design

A cross-sectional study was conducted from September 2015 to December 2016 in three high TB patient load health facilities (Zewditu Memorial hospital, Addis Ketema, and Teklehaimanot Health centers) in Addis Ababa, Ethiopia. A total of 150 volunteer PTB suspected patients who visited the health facilities during the study period were included as study participants. Of these, 75 (50%) cases were sputum scarce patients and the remaining cases were patients who were able to give sputum for diagnosis. Urine and socio-demographic data were collected from all 150 PTB suspected cases, and sputum specimens were collected from 75 cases who were able to give sputum. Specimen analysis was performed at National TB Reference Laboratory, Ethiopia Public Health Institute (EPHI), Ethiopia.

Specimens Collection and Laboratory Analysis

Early morning urine specimens (50ml) were collected by a sterile container with a tightly fitted lid and 5 to 10 ml sputum specimens were collected using a sterile wide mouth 50 ml falcon tube. All specimens were stored at 2-8 ^{o}C at specimen collection sites until transported to the laboratory using cold chain. The Ziehl-Neelsen (ZN) microscopy was used based on the method described previously (WHO, 1998). For GeneXpert MTB/RIF Analysis, urine specimens were centrifuged at 3000*g* for 15 minutes, and the supernatant decanted then the pellets suspended in 1ml of sterile phosphate-buffered saline. Sample reagent was mixed at a 2:1 ratio with 1 ml of re-suspended urine with vigorously shaking for 10 times, and then incubated at room temperature for 10 minutes and again shaking vigorously, and incubated at room temperature for 5 min (Peter *et al.,* 2012).

For sputum specimen analysis, 2ml of specimen reagent buffer was added into 1 ml of the sputum specimens. After 15 minutes of incubation with intermittent hand mixing. Finally, 2 ml of the processed specimen was added into the cartridge and the cartridge was placed on the GeneXpert machine, and results are recorded within 2 hours as MTB-negative or positive and RIF sensitive or resistant (Barnard *et al.,* 2012, Theron *et al.,* 2011).

Urine and sputum specimens were cultured using Lowenstein-Jensen (LJ) solid media and Mycobacteria Growth Indicator Tube (MGIT) test as following urine and sputum specimens were decontaminated according to the procedure by Petroff method. Briefly, each patient urine specimen was centrifuged at 3000 × *g* for 20 minutes and the supernatant was discarded to get the pellet. The urine pellet and sputum were decontaminated with an equal amount of 4% NaOH and incubated for 15 minutes, and then the suspension was neutralized with phosphate-buffered saline (PBS; pH 6.8) and then centrifuged at 3000 × *g* for 15 minutes. The pellets were re-suspended in PBS; 0.1 ml was inoculated on LJ slants at 37 °C for 8 weeks (Kent and Kubica 1985) while 0.5 ml was inoculated in the tube and loaded into BACTEC™ MGIT 960 instrument for 42 days maximum (Siddiqi and Rüsch, 2006). All positive results were confirmed by using MPT64 antigen detection (CapiliaTB) (ECDC, 2016). AS described by BD Diagnostics, drug susceptibility testing against first-line anti-TB drugs rifampicin (RIF), isoniazid (INH), streptomycin (SM), and ethambutol (EMB) were performed with the Bactec MGIT 960 method with the standard concentration 1.0 µg/ml for RIF, 0.1 µg/ml for INH, 5.0 µg/ml for EMB and 1.0 µg/ml for STR (Siddiqi and Rüsch, 2006).

Data Analysis

Data were entered using Microsoft Excel and analyzed using SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Descriptive analysis was done to characterize the study population, and diagnostic sensitivity and specificity of GeneXpert MTB/RF Assay were determined using culture results as reference test with 95% confidence intervals (CI) at α=0·05 (Agresti and Coull, 1998).

Ethical Considerations

Ethical approval was obtained from Research and Ethical Review Committee of the Addis Ababa University. The Study subjects were told about the benefit of being tested. Besides, a written and or oral informed consent was taken from each study participant. Permission was also obtained from study sites and data and specimens were collected and analyzed using codes for confidentiality purpose throughout the study period.

6.4 Results

Socio-Demography Data

A total of 150 PTB suspected cases participated in this study, and 93(62%) of the participants were males and 57 (38%) participants were females. The age of 102 (68%) participants was between 25 and 44 with the mean age of 35.4 years. Concerning their occupation, majority 40 (26.7%) of participants were self-employed and 38 (25.3%) were working for a private organization. A majority, 93 (92.7%) participant were urban residents (Table 6.1).

Regarding TB related condition, 93 (62%) cases were TB/HIV co-infected, and 17 (11.3%) case were previously TB treated cases, and among previously treated cases, a total of 15/17 (88.2%) cases were TB/HIV co-infected. Eighteen (12.0%) cases reported that they were regular alcohol users and only one participant was a regular smoker (Table 6.2).

Diagnostic Performance of GeneXpert MTB/RIF

Urine specimens from 150 cases were subjected to smear, culture, and GeneXpert MTB/RIF examinations. Of these, 6(4.0%) urine specimens were positive by GeneXpert MTB/RIF, of which 5/6(83.3%) urine were also culture-positive (LJ and BACTEC™ MGIT 960). Likewise, 75 sputum specimens were analyzed and 26 (34.6%) specimens were positive by GeneXpert MTB/RIF and culture (LJ and BACTEC™ MGIT 960); among 26 sputum positive cases, 1 (3.8%) case was also positive for urine by GeneXpert MTB/RIF and cultures.

Table 6-1: Socio-demographic characteristics of PTB suspected cases reported to health facilities in Addis Ababa Ethiopia (n=150) January, 2017

Variable		Number (%)
Sex	Female	57 (38%)
	Male	93 (62%)
Age Group	15-24	20 (13.3)%
	25-34	58 (38.7%)
	35-44	44 (29.3%)
	45-54,	20 (13.3)%
	55-64	3 (2%)
	>65	5 (3.3)%
Marital status	Married	89 (59.4%)
	Single	43 (28.6%)
	Divorced	10 (6.7%)
	Widowed	8 (5.3%)
Region	Addis Ababa	144 (96.0%)
	Oromia	5 (3.3%)
	SNNPR	1 (0.7%)
Residence	Rural	11 (7.3%)
	Urban	93 (92.7%)
Occupation	Civil servant	21 (14%)
	Daily laborer	24 (16%)
	House wife	22 (14.6%)
	Self Employed	40 (26.7%)
	Private org	38 (25.3%)
	Unemployed	5 (3.3%)

SNNPR: Southern Nations, Nationalities, and Peoples' Region

Moreover among sputum culture negative cases, one case was sputum positive for GeneXpert MTB/RIF only in addition to this among smear-negative cases (n=61), 1 (1.6%) case was positive for GeneXpert MTB/RIF and cultures for urine only. Regarding to drug sensitivity tests, all isolates were susceptible to all first-line drugs (RIF, INH, EMB, and STR).

Table 6-2: Clinical history and laboratory test results of PTB suspected cases reported to health facilities in Addis Ababa Ethiopia (n=150)

Health condition		Cases Number (%)	Urine positive		Sputum positive		Sputum ZN Positive (n=14)	Urine ZN Positive (n=1)	Urine and Sputum positive**(n=1)	Urine Positive with Sputum Negative*** (n=1)
			Culture* (n=5)	GeneXpert (n=6)	Culture* (n=26)	GeneXpert (n=27)				
TB History	New	133 (88.7%)	3 (60%)	4 (66.7%)	19 (73.1%)	20 (74.1%)	11 (78.6%)	1 (100%)	0 (0.0%)	1 (100.0%)
	Retreatment	17 (11.3%)	2 (40%)	2 (33.3%)	7 (26.9%)	7 (25.9%)	3 (21.4%)	0 (0.0%)	1 (100%)	0 (0.0%)
HIV status	Negative	54 (36.0%)	1 (20%)	1 (16.7%)	9 (34.6%)	9 (33.3%)	7 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Positive	93 (62.0%)	4 (80%)	5 (83.3%)	16 (61.5%)	17 (63.0%)	7 (50.0%)	1 (100%)	1 (100%)	1 (100%)
	Unknown	3 (2.0%)	0 (0%)	0 (0.0%)	1 (3.8%)	1 (3.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Smoking	Yes	1 (0.7%)	0 (0%)	0 (0.0%)	1 (3.8%)	1 (3.7%)	1 (7.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	No	149 (99.3%)	5 (100%)	6 (100%)	25 (96.2%)	26 (96.3%)	13 (92.9%)	1 (100%)	1 (100%)	1 (100%)
Alcohol drinking	Yes	18 (12.0%)	2 (40%)	2 (33.3%)	3 (11.5%)	3 (11.1%)	1 (7.1%)	0 (0.0%)	1 (100%)	1 (100%)
	No	132 (88.0%)	3 (60%)	4 (66.7%)	23 (88.5%)	24 (88.5%)	13 (92.9%)	1 (100%)	0 (0.0%)	0 (0.0%)
Patient with Sputum	Yes	75 (50.0%)	1 (20%)	2 (33.3%)	26 (100%)	26 (100%)	14 (100%)	NA	1 (100%)	1 (100%)
	No	75 (50.0%)	4 (80%)	4 (66.7%)	NA	NA	NA	NA	NA	NA
Patient with Urine	Yes	150 (50.0%)	5 (100%)	6 (100%)	NA	NA	1 (7.1%)	1 (100%)	1 (100%)	1 (100%)
	No	0 (0.0%)	NA	NA	NA	NA	NA	NA	NA	NA

ZN: Ziehl-Neelsen; NA: Not Applicable

* Culture: both Lowenstein Jensen (LJ) and Mycobacteria Growth Indicator cultures (MGIT)

**Urine and Sputum were positive by GenXpert and Culture (LJ and MGIT),

*** Sputum was negative for smear and culture but urine was positive by GenXpert and Cultures (LJ and MGIT),

Among GeneXpert MTB/RIF positive urine (n=6), a total of 4 (66.7%) urine were collected from patients who were unable to produce sputum (sputum scarce), and all the cases were also TB/HIV co-infected. Moreover, among culture and GeneXpert MTB/RIF positive urine specimens (n=5), 4 (80%) specimens were also collected from TB/HIV co-infected cases, and 2 (40%) specimens were from previously treated cases. In addition, out of 26 culture and GeneXpert MTB/RIF positive sputum, 16(61.5%) sputum were collected from HIV positive cases and 19(73.1%) were from new TB cases. Moreover, 11 (42.3%) microscopy positive sputum specimens were from new TB cases. A case with both urine and sputum positive and a case with urine positive but smear and culture negative were HIV positive, and both these cases were previously treated cases (Table 6.2).

Table 6-3: Comparison of GeneXpert MTB/RIF® and LJ and BACTEC™ MGIT 960 cultures using urine and sputum specimens

		Reference/Standards Method (LJ and MGIT)					
		Urine Culture (n=150)			**Sputum Culture (n=75)**		
		Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**
GeneXpert MTB/RIF®	**Positive**	**5**	1	**6**	**26**	1	**27**
	Negative	**0**	144	**144**	**0**	48	**48**
	Total	**5**	145	**150**	**26**	49	**75**

LJ: Lowenstein Jensen; MGIT: Mycobacteria Growth Indicator Tube

A summary of the performance data is shown in table 6.3, a total of 5 (3.3%) urine specimens were positive by LJ and BACTEC™ MGIT 960 cultures, resulting in 100% sensitivity with the GeneXpert MTB/RIF assay, similarly, the GeneXpert MTB/RIF had specificity of 99.3% (95%CI: 97.4-100%) using LJ and BACTEC™ MGIT 960 cultures as reference test. Regarding to sputum, 26 (34.6%) sputum were positive by both cultures, resulting in 100% sensitivity and 98.0% (95% CI: 94.8-100%) specificity for GeneXpert MTB/RIF assay. The overall performance agreement between the GeneXpert MTB/RIF assay and LJ and BACTEC™ MGIT 960 cultures using urine

and sputum specimens were 99.3% (95% CI: 97.4-100%) and 98.7% (95% CI: 96.1-100%) respectively.

Table 6-4: Comparison of ZN Microscopy with GeneXpert MTB/RIF® and LJ and BACTEC™ MGIT 960 cultures using sputum specimens (n=75)

		Reference/Standards Method					
		GeneXpert MTB/RIF®			LJ and MGIT culture		
		Positive	Negative	Total	Positive	Negative	Total
ZN Method (Microscopy)	Positive	14	0	14	14	0	14
	Negative	13	48	61	12	49	61
	Total	27	48	75	26	49	75

LJ: Lowenstein Jensen; MGIT: Mycobacteria Growth Indicator Tube ZN: Ziehl-Neelsen

Moreover, the sensitivity of ZN microscopy for culture-confirmed tuberculosis was 53.9% (95% CI: 42.6- 65.2%) and with 100% specificity. With regard to GeneXpert MTB/RIF sputum positive, the sensitivity of the ZN microscopy test was 51.9% (95% CI: 40.7-63.3%) and 100% specificity. Furthermore, the overall performance agreement of ZN microscopy with GeneXpert MTB/RIF assay and cultures by using sputum specimens was found to be 82.7% (95% CI: 74.1-91.3%) and 84.0% (95% CI: 75.7-92.3%) respectively (Table 6.4). In general, the cumulative total of TB confirmed cases among the TB suspects increased from 26 (17.3%) to 30 (20.0%) by using urine as an alternative specimen.

6.5 Discussion

Ethiopia is one of the 30th high burden countries (TB, TB/HIV, and MDR-TB), and these countries account for about 90% of the global burden and the high burden of HIV-associated TB is found in Africa region (WHO, 2017). But a diagnosis of TB from people living with HIV is a major diagnostic challenge; about one-third of TB-HIV co-infected patients fail to produce a sputum specimen (Peter *et al.,* 2012) so early diagnosis and treatment of TB are delayed. This is the most challenge especially for resource-poor countries where infrastructure, cost of equipment and reagents, and

lack of competent professionals are the major concerns including high TB/HIV prevalence. Fortunately, the use of GeneXpert MTB/RIF assay has expanded substantially because of short turnaround time and WHO recommendation to use it as an initial diagnostic test for TB. However, obtaining good quality sputum specimen or other pulmonary specimens from HIV-TB co-infected and children is always difficult.

In our study, we evaluated the performance of GeneXpert MTB/RIF assay using urine as an alternative specimen for diagnosing pulmonary TB and this study showed that 4% of urine was positive for *M. tuberculosis*. This finding is consistent with several studies that showed *M. tuberculosis* excretion in urine, and recently several studies also revealed that PTB patients had TB positive urine (Torrea *et al.,* 2005, Gopinath *et al.,* 2007, Peter *et al.,* 2012, Rebollo *et al.,* 2006). Besides, in comparison with the cultures, GeneXpert MTB/RIF assay detection rate was higher than cultures, this could be due to the ability of GeneXpert MTB/RIF to detect the dead bacilli from specimens; a similar detection rate was reported from South Africa (Peter *et al.,* 2012) India (Gopinath *et al.,* 2007), Burkina Faso (Rebollo *et al.,* 2006) and Spain (Torrea *et al.,* 2005).

Moreover in our findings, one case was urine positive with the GeneXpert MTB/RIF assay but the culture result remained negative, it could be due to presence of dead bacilli in the urine specimen and poor specimen handling mechanism however similar study done by Hillemann and colleague also showed that seven culture negative patients were positive with GeneXpert MTB/RIF and finally the majority the cases were confirmed as TB patients (Hillemann *et al.,* 2011). Among sputum positive patients, 3.8% were positive for urine and sputum by GeneXpert MTB/RIF and culture methods. Likewise, a study done in India showed that 26.1% of the cases were MTB positive for urine and sputum, this is higher than our study. It is also possible that *M. tuberculosis* or its fragments may or may not have been excreted during the time of specimen collection (Missirliu *et al.,* 1996). Moreover, endogenous PCR inhibitors like acidic polysaccharides, glycoproteins, urea, and unidentified non-proteinaceous DNA-associated substances cause

chelation of free magnesium ions and are known to inhibit the PCR amplification (Monteiro *et al.,* 1997). However, we were not able to find published data about performance between urine and sputum using GeneXpert MTB/RIF methods.

In our study, the sensitivity of the GeneXpert MTB/RIF test using urine was found to be 100%. This finding was consistent with a study conducted by Hilleman *et al.,* that the GeneXpert MTB/RIF sensitivity was 100% with urine specimens (Hillemann *et al.,* 2011). With regard to the specificity, our study showed that the specificity of GeneXpert MTB/RIF test was 99.3% which is comparable with the study done in South Africa on HIV-infected patients who are smear-negative and sputum scarce found with 98% of specificity. Similarly, our study also focused on sputum scarce patients, and we found the higher urine GeneXpert MTB/RIF positive result from sputum scarce patients. Moreover, a significant association between HIV patient and urine MTB positivity was found in this study. It might be indicating dissemination and increasing load of MTB in immune-compromised patients (Peter *et al.,* 2012).

Further, sputum scarce and smear-negative patients constitute a major burden of undiagnosed TB (Cox *et al.,* 2010). Misdiagnosis and diagnostic delays lengthen hospital stay and delay rapid treatment initiation, likely worsening TB related morbidity and mortality (Holtz *et al.,* 2011). In sputum-scarce or smear and culture negative cases, the alternative specimens are bronchoalveolar lavage and gastric lavage fluids, but these methods need high skilled professionals with special equipment, and also they are painful techniques, in addition to this these specimens do not add significant advantage for diagnostic of TB (Gopinath *et al.,* 2007). However, our study showed an additional 15.4% of PTB cases using urine by GeneXpert MTB/RIF Assay and this finding is highly significant and encouraging for TB control program. Thus, urine specimens could contribute for improvement of PTB diagnosis, particularly for sputum scarce patients.

Limitations of the study

Although this research was carefully prepared, it has some important limitations. The study subjects unable to generate a sputum sample was small, and a majority of the sputum-scarce patients were disproportionately recruited in the selected health facilities. Moreover, even though we recruited all cases according to signs and symptoms of pulmonary TB, there might be cases with additional extra-pulmonary tuberculosis in urinary tract system.

6.6 Conclusions

In conclusion, our study revealed that urinary GeneXpert MTB/RIF test increase the detection rate of pulmonary TB among sputum scarce patients. As laboratory diagnosis and treatment of TB remains weak in most resource-poor countries, the development of sensitive and rapid diagnostic method or other appropriate and easy to collect alternative specimen is crucial. Therefore, the finding of our study leads us to suggest that urine specimen would be an alternative specimen for diagnosis of active pulmonary TB among sputum scarce patients using GeneXpert assay. Moreover, as urine is an easily available specimen, it might minimize the use of more invasive techniques for collecting specimens from bronchoalveolar and gastric for diagnosis of PTB.

Chapter 7

General Conclusion and Recommendation

7.1 General Conclusion, and Recommendation

This study has revealed that the prevalence of MDR-TB in the study area was higher compared to WHO data and previous studies done in Ethiopia and that the proportion of MDR-TB among previously treated patients and young age group was also higher than previous studies. The major risk factors for the development of MDR-TB were TB/HIV co-infection, frequent cigarette smoking, frequent consumption of alcohol, hospital admission, and a history of visits to health facilities. The study indicated that there is a progressive MDR-TB transmission particularly in the productive age group of the population. Actions should be taken to improve TB control program to the population at risk of MDR-TB if Ethiopia is to avoid an environment in which MDR-TB continues to increase its impact on the health of the nation.

Moreover, the highest rate of mutations associated with drug resistance was found at codons 531, 315, 306, 65, and 88 in rpoB, katG, embB, pncA, and rpsl genes respectively. In addition, the majority of the MDR-TB strains had a mutation at codon 306, and this mutation found to be an important molecular indicator of EMB resistance and MDR-TB. Despite the absence of a mutation at codon 306 does not rule out phenotypic EMB susceptibility and non MDR-TB. In addition, a mutation was found 5 codons far way from 81 bp RRDR at codon 538 and also at codon 533 with RIF susceptible in RRDR, so it is very important to screen MDR-TB suspected patient using other methods than the GeneXpert assay. Besides, the patterns and diversity of mutations in drug target genes of *M. tuberculosis* isolates from Ethiopia is similar to that of the majority of *M. tuberculosis* isolates prevalent globally. This may have important implications for the roll-out of rapid molecular tests to identify drug-resistant MTB strains, but, there should be a system for continuous monitoring the patterns of drug-resistance mutations to ensure that if clonal groups of MTB do emerge. AS this study is the first study done to identify mutations associated with resistance in *M. tuberculosis* complex isolates from Ethiopia by sequencing the drug target genes, the study points out that there were discrepant results between phenotypic drug susceptibility and molecular

tests. Although there are similar reports from different countries, our study suggests that further studies should be done to investigate the main causes associated with discordant results.

In this study, there was a highly diverse population structure of *M. tuberculosis isolates*, and the predominance strain was the Dehli/CAS lineage and ranked as the second predominant among MDR-TB strains. Likewise, the highest the predominant genotype among the MDR-TB isolates was TUR lineage with more than 46%, and there was a statistically significant association between TUR strain infection and multidrug resistance. Moreover, there was a high clustering and recent transmission rate among drug-resistant strains. In conclusion, the high rate of transmission and clustering underlines an active transmission of MDR-TB strains in the population

Furthermore, the study depicted that a urinary GeneXpert MTB/RIF test increases the detection rate of pulmonary TB among sputum scarce patients. As laboratory diagnosis and treatment of TB remains weak in most resource-poor countries, the development of sensitive and rapid diagnostic method or other appropriate and easy to collect alternative specimen is crucial. Therefore, the finding of our study leads us to suggest that urine specimen would be an alternative specimen for diagnosis of active pulmonary TB among sputum scarce patients using GeneXpert assay. Moreover, as urine is an easily available specimen, it might minimize the use of more invasive techniques for collecting specimens from bronchoalveolar and gastric lavagefor diagnosis of PTB.

Altogether, the recommendation would be that TB patients suspected of MDR-TB should be identified in a timely manner and treated according to the treatment guideline, and the country should focus its efforts on designing and developing a strategy towards early detection and treatment of MDR-TB cases in the population, and monitoring systems to investigate the trend of MDR-TB incidence and efficacy of MDR-TB treatment regimens. Moreover, as the high rate of transmission index indicates an active transmission of MDR-TB strains in the population, further

studies should be done to determine the transmission dynamics and *M. tuberculosis* lineages associated with drug resistance that prevalent in the country. In addition to this, studies devoted to increasing and refining the public health community's understanding of risk factors for the development of MDR-TB in the population should also be part of the TB control program. In summary, this emphasizes the importance of strengthening the health system in order to improve the TB control and prevention program at large.

References

Aceti, A., Zanetti, S., Mura, M.S., Sechi, L.A., Turrini, F., Saba, F., Babudieri, S., Mannu, F. and Fadda, G. (1999). Identification of HIV patients with active pulmonary tuberculosis using urine based polymerase chain reaction assay. Thorax, 54(2):145-146.

Achtman, M., 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annual Review of Microbiology, 62:53-70.

Adane Mihret, Yonas Bekele, Milikit Aytenew, Meseret Abebe, Liya Wassie, Loxton, G.A., Yamuah, L., Abraham Aseffa, Walzl, G. and Howe, R. (2012). Modern lineages of Mycobacterium tuberculosis in Addis Ababa, Ethiopia: implications for the tuberculosis control programe. African health sciences, 12(3):339-344.

Adeeb Shehzada, Gauhar Rehmana, Mazhar Ul-Islamb, Waleed Ahmad Khattakb, and Young Sup Leea (2013). Challenges in the development of drugs for the treatment of tuberculosis. Brazilian Journal of Infectious Diseases, 17(1):74–78.

Agresti, A. and Coull, B.A. (1998). Approximate is better than “exact” for interval estimation of binomial proportions. The American Statistician, 52(2):119-126.

Al-Hajoj, S., Varghese, B., Al-Habobe, F., Shoukri, M.M., Mulder, A. and van Soolingen, D. (2013). Current trends of Mycobacterium tuberculosis molecular epidemiology in Saudi Arabia and associated demographical factors. Infection, Genetics and Evolution 16:362-368.

Al-Hajoj, S.A. (2010). Tuberculosis in Saudi Arabia: can we change the way we deal with the disease? Journal of infection and public health 3(1):17-24.

Allix-Béguec, C., Fauville-Dufaux, M., Stoffels, K., Ommeslag, D., Walravens, K., Saegerman, C. and Supply, P. (2010). Importance of identifying Mycobacterium bovis as a causative agent of human tuberculosis. European Respiratory Journal 35(3): 692-694.

Allix-Béguec, C., Harmsen, D., Weniger, T., Supply, P. and Niemann, S. (2008). Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. Journal of clinical microbiology 46(8):2692-2699.

Amare Deribew , Nebiyu Negussu, Zenebe Melaku, Kebede Deribe (2011). Investigation outcomes of tuberculosis suspects in the health centers of Addis Ababa, Ethiopia. PLoS One 6(4):e18614.

Amare Deribew, Gemeda Abebe, Ludwig Apers, Alemseged Abdissa, Fetene Deribe,

Kifle Woldemichael, Chali Jira, Markos Tesfaye, Jafar Shiffa, Abraham Aseffa, Mesele Bezabih, Tadiye Abeje and Robert Colebunders (2012). Prevalence of pulmonary TB and spoligotype pattern of Mycobacterium tuberculosis among TB suspects in a rural community in Southwest Ethiopia. BMC infectious diseases 12(1):54.

Andres S., Hillemann D., R¨usch-Gerdes S, Richter E. (2014). Occurrence of rpoB mutations in isoniazid-resistant but rifampin-susceptible Mycobacterium tuberculosis isolates from Germany. Antimicrob Agents Chemother. 58:590–592.

Asres Berhan, Yifru Berhan, Desalegn Yizengaw (2013). A meta-analysis of drug-resistant tuberculosis in Sub-Saharan Africa: how strongly associated with previous treatment and HIV co-infection?. Ethiopian journal of health sciences 23(3):271-282.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K.S., Wilson, T., Collins, D., de Lisle, G. and Jacobs, W.R. (1994). inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science, 263(5144):227-230.

Banu, S., Rahman, M.T., Uddin, M.K.M., Khatun, R., Khan, M.S.R., Rahman, M.M., Uddin, S.I., Ahmed, T. and Heffelfinger, J.D. (2015). Effect of active case finding on prevalence and transmission of pulmonary tuberculosis in Dhaka Central Jail, Bangladesh. PloS One, 10(5):e0124976.

Barlett, J.E., Kotrlik, J.W. and Higgins, C.C. (2001). Organizational research: Determining appropriate sample size in survey research. Information technology, learning, and performance journal 19(1):43.

Barnard, M., van Pittius, N.G., Van Helden, P.D., Bosman, M., Coetzee, G. and Warren, R.M. (2012). The diagnostic performance of the GenoType MTBDRplus version 2 line probe assay is equivalent to that of the Xpert MTB/RIF assay. Journal of clinical microbiology 50(11):3712-3716.

Barroso, E.C., Mota, R.M.S., Santos, R.O., Sousa, A.L.O., Barroso, J.B. and Rodrigues, J.L.N. (2003). Risk factors for acquired multidrug-resistant tuberculosis. Jornal de Pneumologia 29(2):89-97.

Behr, M.A., Warren, S.A., Salamon, H., Hopewell, P.C., De Leon, A.P., Daley, C.L. and Small, P.M. (1999). Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. The Lancet 353(9151):444-449.

Belay Tessema, Beer, J., Emmrich, F., Sack, U. and Rodloff, A.C. (2012). First-and second-line anti-tuberculosis drug resistance in Northwest Ethiopia. The International Journal of Tuberculosis and Lung Disease 16(6):805-811.

Belay Tessema,, Beer, J., Merker, M., Emmrich, F., Sack, U., Rodloff, A.C. and Niemann, S.

(2013). Molecular epidemiology and transmission dynamics of Mycobacterium tuberculosis in Northwest Ethiopia: new phylogenetic lineages found in Northwest Ethiopia. BMC infectious diseases 13(1):131.

Bhanu, N.V., Van Soolingen, D., Van Embden, J.D.A., Dar, L., Pandey, R.M. and Seth, P. (2002). Predominace of a novel Mycobacterium tuberculosis genotype in the Delhi region of India. Tuberculosis 82(2-3):105-112.

Bhuju, S., de Souza Fonseca, L., Marsico, A.G., de Oliveira Vieira, G.B., Sobral, L.F., Stehr, M., Singh, M. and Saad, M.H.F. (2013). Mycobacterium tuberculosis isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the pncA gene. Infection, Genetics and Evolution, 19:1-6.

Bishop, K., Blumberg, L., Trollip, A., Smith, A., Roux, L., York, D. and Kiepiela, P. (2001). Characterisation of the pncA gene in Mycobacterium tuberculosis isolates from Gauteng, South Africa. The International Journal of Tuberculosis and Lung Disease 5(10):952-957.

Bishop, P.J. and Neumann, G. (1970). The history of the Ziehl-Neelsen stain. Tubercle, 51(2):196-206.

Bolotin, S., Alexander, D.C., Chedore, P., Drews, S.J. and Jamieson, F. (2009). Molecular characterization of drug-resistant Mycobacterium tuberculosis isolates from Ontario, Canada. Journal of antimicrobial chemotherapy 64(2):263-266.

Brittle, W., Marais, B.J., Hesseling, A.C., Schaaf, H.S., Kidd, M., Wasserman, E. and Botha, T. (2009). Improvement in mycobacterial yield and reduced time to detection in pediatric samples by use of a nutrient broth growth supplement. Journal of clinical microbiology 47(5):1287-1289.

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K. and Parsons, L.M. (2002). A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proceedings of the national academy of Sciences, 99(6):3684-3689.

Brossier, F., Veziris, N., Truffot-Pernot, C., Jarlier, V. and Sougakoff, W. (2011). Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy, 55(1):355-360.

Bruchfeld, J., Aderaye, G., Palme, I.B., Bjorvatn, B., Ghebremichael, S., Hoffner, S. and Lindquist, L. (2002). Molecular epidemiology and drug resistance of Mycobacterium tuberculosis isolates from Ethiopian pulmonary tuberculosis patients with and without

human immunodeficiency virus infection. Journal of clinical microbiology, 40(5):1636-1643.

Brudey, K., Filliol, I., Ferdinand, S., Guernier, V., Duval, P., Maubert, B., Sola, C. and Rastogi, N. (2006). Long-term population-based genotyping study of Mycobacterium tuberculosis complex isolates in the French departments of the Americas. Journal of clinical microbiology, 44(1):183-191.

Caminero, J.A. (2004). A tuberculosis guide for specialist physicians. Paris: International Union Against Tuberculosis and Lung Disease, pp. 79.

Campbell, P.J., Morlock, G.P., Sikes, R.D., Dalton, T.L., Metchock, B., Starks, A.M., Hooks, D.P., Cowan, L.S., Plikaytis, B.B. and Posey, J.E. (2011). Molecular detection of mutations associated with first-and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy 55(5):2032-2041.

Camus, J.C., Pryor, M.J., Médigue, C. and Cole, S.T. (2002). Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. Microbiology 148(10):2967-2973.

Carette, X., Blondiaux, N., Willery, E., Hoos, S., Lecat-Guillet, N., Lens, Z., Wohlkönig, A., Wintjens, R., Soror, S.H., Frénois, F. and Dirié, B. (2011). Structural activation of the transcriptional repressor EthR from Mycobacterium tuberculosis by single amino acid change mimicking natural and synthetic ligands. Nucleic acids research 40(7):3018-3030.

Carter, A.P., Clemons, W.M., Brodersen, D.E., Morgan-Warren, R.J., Wimberly, B.T. and Ramakrishnan, V. (2000). Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature, 407(6802):340-348.

Centers for Disease Control and Prevention (CDC) (2009). Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. Morbidity and mortality weekly report, 58(1):7-10.

Chang, K.C., Yew, W.W. and Zhang, Y., 2011. Pyrazinamide susceptibility testing in Mycobacterium tuberculosis: a systematic review with meta-analyses. Antimicrobial agents and chemotherapy 55(10):4499-4505.

Chihota, V.N., Grant, A.D., Fielding, K., Ndibongo, B., Van Zyl, A., Muirhead, D. and Churchyard, G.J. (2010). Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. The international journal of tuberculosis and lung disease 14(8):1024-1031.

Choi, G.E., Jang, M.H., Song, E.J., Jeong, S.H., Kim, J.S., Lee, W.G., Uh, Y., Roh, K.H., Lee,

H.S., Shin, J.H. and Ryoo, N.H. (2010). IS6110-restriction fragment length polymorphism and spoligotyping analysis of Mycobacterium tuberculosis clinical isolates for investigating epidemiologic distribution in Korea. Journal of Korean medical science 25(12):1716-1721.

Colby FH (1961). Essential urology. Williams and Wilkins, Baltimore.

Cole, S.T., Eisenach, K.D., McMurray, D.N. and Jacobs Jr, W.R. (2005). Tuberculosis and the tubercle bacillus. ASM press.

Comas, I., Coscolla, M., Luo, T., Borrell, S., Holt, K.E., Kato-Maeda, M., Parkhill, J., Malla, B., Berg, S., Thwaites, G. and Yeboah-Manu, D. (2013). Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nature Genetics 45(10):1176.

Comas, I., Hailu, E., Kiros, T., Bekele, S., Mekonnen, W., Gumi, B., Tschopp, R., Ameni, G., Hewinson, R.G., Robertson, B.D. and Goig, G.A. (2015). Population genomics of Mycobacterium tuberculosis in Ethiopia contradicts the virgin soil hypothesis for human tuberculosis in Sub-Saharan Africa. Current Biology 25(24):3260-3266.

Comas, I., Homolka, S., Niemann, S. and Gagneux, S. (2009). Genotyping of genetically monomorphic bacteria: DNA sequencing in Mycobacterium tuberculosis highlights the limitations of current methodologies. PloS One 4(11):e7815.

Cooksey, R.C., Morlock, G.P., McQueen, A., Glickman, S.E. and Crawford, J.T. (1996). Characterization of streptomycin resistance mechanisms among Mycobacterium tuberculosis isolates from patients in New York City. Antimicrobial agents and chemotherapy 40(5):1186-1188.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C. and Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Archives of internal medicine, 163(9), pp.1009-1021.

Coscolla M., Gagneux S. (2014). Consequences of genomic diversity in Mycobacterium tuberculosis. Semin Immunol. 26:431–444.

Cox, J.A., Lukande, R.L., Lucas, S., Nelson, A.M., Van Marck, E. and Colebunders, R. (2010). Autopsy causes of death in HIV-positive individuals in sub-Saharan Africa and correlation with clinical diagnoses. AIDS Reviews, 12(4):183-194.

Cuevas-Córdoba, B., Cuellar-Sánchez, A., Pasissi-Crivelli, A., Santana-Álvarez, C.A., Hernández-Illezcas, J. and Zenteno-Cuevas, R. (2013). rrs and rpsL mutations in streptomycin-resistant isolates of Mycobacterium tuberculosis from Mexico. Journal of Microbiology, Immunology and Infection 46(1):30-34.

Cui, Z., Wang, J., Lu, J., Huang, X., Zheng, R. and Hu, Z. (2013). Evaluation of methods for

testing the susceptibility of clinical Mycobacterium tuberculosis isolates to pyrazinamide. Journal of clinical microbiology 51(5):1374-1380.

da Silva, P.E.A., Von Groll, A., Martin, A. and Palomino, J.C. (2011). Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. FEMS Immunology and Medical Microbiology 63(1):1-9.

Dale, J.W., Brittain, D., Cataldi, A.A., Cousins, D., Crawford, J.T., Driscoll, J., Heersma, H., Lillebaek, T., Quitugua, T., Rastogi, N. and Skuce, R.A. (2001). Spacer oligonucleotide typing of bacteria of the Mycobacterium tuberculosis complex: recommendations for standardised nomenclature [The Language of Our Science]. The International Journal of Tuberculosis and Lung Disease 5(3):216-219.

Dalla Costa, E.R., Ribeiro, M.O., Silva, M.S., Arnold, L.S., Rostirolla, D.C., Cafrune, P.I., Espinoza, R.C., Palaci, M., Telles, M.A., Ritacco, V. and Suffys, P.N. (2009). Correlations of mutations in kat G, oxy R-ahp C and inh A genes and in vitro susceptibility in Mycobacterium tuberculosis clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. BMC microbiology 9(1):39.

Daniel Mekonnen Nigus, Wondemagegn Mulu, Bayeh Abera, Aschalew Admassu, Martha Tibebu and Mulat Yimer (2014). Prevalence of multi drug-resistant tuberculosis among presumptive multi drug-resistant tuberculosis cases in Amhara National Regional State, Ethiopia. Journal of Mycobacterium Disease, 4(152):2161-2168.

Dannenberg Jr, A.M. and Rook, G.A. (1994). Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses—dual mechanisms that control bacillary multiplication. In Tuberculosis, pp. 459-483. American Society of Microbiology.

Davis, J.L., Worodria, W., Kisembo, H., Metcalfe, J.Z., Cattamanchi, A., Kawooya, M., Kyeyune, R., den Boon, S., Powell, K., Okello, R. and Yoo, S. (2010). Clinical and radiographic factors do not accurately diagnose smear-negative tuberculosis in HIV-infected inpatients in Uganda: a cross-sectional study. PloS One 5(3):e9859.

Dawit Wolde Meskel, Getahun Abate, Mekuria Lakew, Solomon Goshu, and Abraham Aseffa, (2008). Anti-tuberculosis drug resistance among retreatment patients seen at St Peter Tuberculosis Specialized Hospital. Ethiopian medical journal 46(3):219-225.

De Jong, B.C., Antonio, M. and Gagneux, S. (2010). Mycobacterium africanum—review of an important cause of human tuberculosis in West Africa. PLoS neglected tropical diseases 4(9):e744.

De Jong, B.C., Antonio, M., Awine, T., Ogungbemi, K., De Jong, Y.P., Gagneux, S.,

DeRiemer, K., Zozio, T., Rastogi, N., Borgdorff, M. and Hill, P.C. (2009). Use of spoligotyping and large sequence polymorphisms to study the population structure of the Mycobacterium tuberculosis complex in a cohort study of consecutive smear-positive tuberculosis cases in The Gambia. Journal of clinical microbiology 47(4):994-1001.

Dereje Abate, Taye, B., Abseno, M. and Biadgilign, S., 2012. Epidemiology of anti tuberculosis drug resistance patterns and trends in tuberculosis referral hospital in Addis Ababa, Ethiopia. BMC research notes, 5(1):462.

Dheda, K., Schwander, S.K., Zhu, B., Van, Z.S., Richard, N. and ZHANG, Y. (2010). The immunology of tuberculosis: from bench to bedside. Respirology 15(3):433-450.

Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.L. and Bevins, C.L. (1991). Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proceedings of the National Academy of Science 88(9):3952-3956.

Dickson, S.J., Brent, A., Davidson, R.N. and Wall, R. (2003). Comparison of bronchoscopy and gastric washings in the investigation of smear-negative pulmonary tuberculosis. Clinical infectious diseases 37(12):1649-1653.

Diriba Benti, Berkessa Teferi, Gezahegn Mamo, Tedla Yimer. and Gobona Ameni (2013). Spoligotyping of multidrug-resistant Mycobacterium tuberculosis isolates in Ethiopia. The International Journal of Tuberculosis and Lung Disease 17(2):246-250.

Dorman, S.E. and Chaisson, R.E. (2007). From magic bullets back to the magic mountain: the rise of extensively drug-resistant tuberculosis. Nature medicine 13(3):295.

Doustdar, F., Khosravi, A.D., Farnia, P., Masjedi, M.R. and Velayati, A.A. (2008). Molecular analysis of isoniazid resistance in different genotypes of Mycobacterium tuberculosis isolates from Iran. Microbial Drug Resistance 14(4):273-279.

Drlica, K. and Malik, M. (2003). Fluoroquinolones: action and resistance. Current topics in medicinal chemistry 3(3):249-282.

Dutt, A.K. and Stead, W.W. (1994). Smear-negative pulmonary tuberculosis. In Seminars in respiratory infections 9(2):113-119.

Dye, C. and Williams, B.G. (2010). The population dynamics and control of tuberculosis. Science 328(5980):856-861.

Eldin, G.S.S., Fadl-Elmula, I., Ali, M.S., Ali, A.B., Salih, A.L.G., Mallard, K., Bottomley, C. and McNerney, R. (2011). Tuberculosis in Sudan: a study of Mycobacterium tuberculosis strain genotype and susceptibility to anti-tuberculosis drugs. BMC infectious diseases 11(1):219.

European Centre for Disease Prevention and Control, (ECDC) (2016). Handbook on TB

laboratory diagnostic methods for the European Union. Stockholm.

Falzon, D., Jaramillo, E., Schünemann, H.J., Arentz, M., Bauer, M., Bayona, J., Blanc, L., Caminero, J.A., Daley, C.L., Duncombe, C. and Fitzpatrick, C. (2011). WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update.

Fantahun Biadglegne, Merker, M., Sack, U., Rodloff, A.C. and Niemann, S. (2015). Tuberculous lymphadenitis in Ethiopia predominantly caused by strains belonging to the Delhi/CAS lineage and newly identified Ethiopian clades of the Mycobacterium tuberculosis complex. PloS One 10(9):e0137865.

Farer, L.S., Lowell, A.M. and Meador, M.P. (1979). Extrapulmonary tuberculosis in the United States. American journal of epidemiology 109(2):205-217.

Fauci, A.S. and NIAID Tuberculosis Working Group (2008). Multidrug-resistant and extensively drug-resistant tuberculosis: the National Institute of Allergy and Infectious Diseases Research agenda and recommendations for priority research. The Journal of infectious diseases 197(11):1493-1498.

Faustini, A., Hall, A.J. and Perucci, C.A. (2006). Risk factors for multidrug-resistant tuberculosis in Europe: a systematic review. Thorax 61(2):158-163.

Central Statistical Agency (CSA) (2013). Population Projection of Ethiopia for All Regions at Wereda Level from 2014 – 2017. CSA, Addis Ababa, Ethiopia. Available at: www.csa.gov.et/ehioinfo-internal/

Federal Ministry of Health (FMOH) (2012). Training material on Programmatic management of Drug resistant Tuberculosis in Ethiopia for GHWs Participants' Manual. FDREMoH Addis Ababa, Ethiopia

Federal Ministry of Health (FMOH) (2009). Guideline for program and clinical management of drug-resistant tuberculosis. FMOH. Addis Ababa; Ethiopia

Feleke Mekonnen, Belay Tessema, Feleke Moges, Aschalew Gelaw, Setegn Eshetie and Gemechu Kumera (2015). Multidrug-resistant tuberculosis: prevalence and risk factors in districts of metema and west armachiho, Northwest Ethiopia. BMC infectious diseases 15(1):461.

Fenner, L., Malla, B., Ninet, B., Dubuis, O., Stucki, D., Borrell, S., Huna, T., Bodmer, T., Egger, M. and Gagneux, S. (2011). "Pseudo-Beijing": evidence for convergent evolution in the direct repeat region of Mycobacterium tuberculosis. PloS One 6(9):e24737.

Finken, M., Kirschner, P., Meier, A., Wrede, A. and Böttger, E.C. (1993). Molecular basis of

streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Molecular microbiology 9(6):1239-1246.

Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, Gadisa E, Kiros T, Habtamu M, Hussein J, Zinsstag J (2013). Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerging infectious diseases 19(3):460.

Gagneux, S., DeRiemer, K., Van, T., Kato-Maeda, M., De Jong, B.C., Narayanan, S., Nicol, M., Niemann, S., Kremer, K., Gutierrez, M.C. and Hilty, M. (2006). Variable host–pathogen compatibility in Mycobacterium tuberculosis. Proceedings of the National academy of Sciences of the United States of America 103(8):2869-2873.

Galagan, J.E. (2014). Genomic insights into tuberculosis. Nature Reviews Genetics 15(5):307-320.

Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J. and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet 368(9547):1575-1580.

Garcia de Viedma, D., Lorenzo, G., Cardona, P.J., Alonso Rodriguez, N., Gordillo, S., Ruiz Serrano, M.J. and Bouza, E. (2005). Association between the infectivity of Mycobacterium tuberculosis strains and their efficiency for extrarespiratory infection. The Journal of infectious diseases 192(12):2059-2065.

Gillespie, S.H. (2002). Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective. Antimicrobial agents and chemotherapy 46(2):267-274.

Godreuil, S., Renaud, F., Choisy, M., Depina, J.J., Garnotel, E., Morillon, M., Van de Perre, P. and Banuls, A.L. (2010). Highly structured genetic diversity of the Mycobacterium tuberculosis population in Djibouti. Clinical Microbiology and Infection 16(7):1023-1026.

Gopinath, K. and Singh, S. (2009). Urine as an adjunct specimen for the diagnosis of active pulmonary tuberculosis. International Journal of Infectious Diseases 13(3):374-379.

Gopinath, K., Manisankar, M., Kumar, S. and Singh, S. (2007). Controlling multidrug-resistant tuberculosis in India. The Lancet 369(9563):741-742.

Gutierrez, M.C., Brisse, S., Brosch, R., Fabre, M., Omaïs, B., Marmiesse, M., Supply, P. and Vincent, V. (2005). Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS pathogens 1(1):e5.

Hall, L. and Roberts, G.D. (2006). Non-molecular identification of nontuberculous

mycobacteria in the clinical microbiology laboratory: What's the real deal?. Clinical Microbiology Newsletter 28(10):73-80.

Hanif, M., Malik, S. and Dhingra, V.K. (2009). Acquired drug resistance pattern in tuberculosis cases at the State Tuberculosis Centre, Delhi, India. The International Journal of Tuberculosis and Lung Disease 13(1):74-78.

Hazbón, M.H., Brimacombe, M., del Valle, M.B., Cavatore, M., Guerrero, M.I., Varma-Basil, M., Billman-Jacobe, H., Lavender, C., Fyfe, J., García-García, L. and León, C.I. (2006). Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy 50(8):2640-2649.

Hazbón, M.H., del Valle, M.B., Guerrero, M.I., Varma-Basil, M., Filliol, I., Cavatore, M., Colangeli, R., Safi, H., Billman-Jacobe, H., Lavender, C. and Fyfe, J. (2005). Role of embB codon 306 mutations in Mycobacterium tuberculosis revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. Antimicrobial agents and chemotherapy 49(9):3794-3802.

Hegde, S.S., Vetting, M.W., Roderick, S.L., Mitchenall, L.A., Maxwell, A., Takiff, H.E. and Blanchard, J.S. (2005). A fluoroquinolone resistance protein from Mycobacterium tuberculosis that mimics DNA. Science 308(5727):1480-1483.

Helal, Z.H., Ashour, M.S.E.D., Eissa, S.A., Abd-Elatef, G., Zozio, T., Babapoor, S., Rastogi, N. and Khan, M.I. (2009). Unexpectedly high proportion of ancestral Manu genotype Mycobacterium tuberculosis strains cultured from tuberculosis patients in Egypt. Journal of Clinical Microbiology 47(9):2794-2801.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. (1997). Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23(3):504-511.

Hepple, P., Ford, N. and McNerney, R. (2012). Microscopy compared to culture for the diagnosis of tuberculosis in induced sputum samples: a systematic review. The international journal of tuberculosis and lung disease 16(5):579-588.

Herrera, L., Jiménez, S., Valverde, A., García-Aranda, M.A. and Sáez-Nieto, J.A. (2003). Molecular analysis of rifampicin-resistant Mycobacterium tuberculosis isolated in Spain (1996–2001). Description of new mutations in the rpoB gene and review of the literature. International journal of antimicrobial agents 21(5):403-408.

Hershberg, R., Lipatov, M., Small, P.M., Sheffer, H., Niemann, S., Homolka, S., Roach, J.C.,

Kremer, K., Petrov, D.A., Feldman, M.W. and Gagneux, S. (2008). High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography. PLoS biology 6(12):e311.

Heym, B., Honore, N., Schurra, C., Cole, S.T., Truffot-Pernot, C., Grosset, J.H., Banerjee, A., Jacobs, W.R. and Van Embden, J.D.A. (1994). Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. The Lancet 344(8918):293-298.

Hillemann, D., Kubica, T., Agzamova, R., Venera, B., Rüsch-Gerdes, S. and Niemann, S. (2005). Rifampicin and isoniazid resistance mutations in Mycobacterium tuberculosis strains isolated from patients in Kazakhstan. The International Journal of Tuberculosis and Lung Disease 9(10):1161-1167.

Hillemann, D., Rüsch-Gerdes, S. and Richter, E. (2009). Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens. Journal of clinical microbiology 47(6):1767-1772.

Hillemann, D., Rüsch-Gerdes, S., Boehme, C. and Richter, E. (2011). Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. Journal of clinical microbiology 49(4):1202-1205.

Hirano, K., Takahashi, M., Kazumi, Y., Fukasawa, Y. and Abe, C. (1998). Mutation in pncA is a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis. Tubercle and Lung Disease 78(2):117-122.

Hoek, K.G., Schaaf, H.S., Gey van Pittius, N.C., van Helden, P.D. and Warren, R.M. (2009). Resistance to pyrazinamide and ethambutol compromises MDR/XDR-TB treatment. SAMJ: South African Medical Journal 99(11):785-787.

Holtz, T.H., Kabera, G., Mthiyane, T., Zingoni, T., Nadesan, S., Ross, D., Allen, J., Chideya, S., Sunpath, H. and Rustomjee, R. (2011). Use of a WHO-recommended algorithm to reduce mortality in seriously ill patients with HIV infection and smear-negative pulmonary tuberculosis in South Africa: an observational cohort study. The Lancet infectious diseases 11(7):533-540.

Homolka, S., Meyer, C.G., Hillemann, D., Owusu-Dabo, E., Adjei, O., Horstmann, R.D., Browne, E.N., Chinbuah, A., Osei, I., Gyapong, J. and Kubica, T. (2010). Unequal distribution of resistance-conferring mutations among Mycobacterium tuberculosis and Mycobacterium africanum strains from Ghana. International Journal of Medical Microbiology 300(7):489-495.

Hoshide, M., Qian, L., Rodrigues, C., Warren, R., Victor, T., Evasco, H.B., Tupasi, T., Crudu, V. and Douglas, J.T. (2013). Geographical differences associated with SNPs in nine gene targets among resistant clinical isolates of Mycobacterium tuberculosis. Journal of Clinical Microbiology JCM-00857.

Hou, L., Osei-Hyiaman, D., Zhang, Z., Wang, B., Yang, A. and Kano, K. (2000). Molecular characterization of pncA gene mutations in Mycobacterium tuberculosis clinical isolates from China. Epidemiology and Infection 124(2):227-232.

Htike, K.P.M., Pitaksajjakul, P., Tipkrua, N., Wongwit, W., Jintaridh, P. and Ramasoota, P. (2014). Novel mutation detection in rpoB of rifampicin-resistant mycobacterium tuberculosis using pyrosequencing. Southeast Asian Journal of Tropical Medicine and Public Health 45(4):843.

Huard, R.C., Fabre, M., de Haas, P., Lazzarini, L.C.O., van Soolingen, D., Cousins, D. and Ho, J.L. (2006). Novel genetic polymorphisms that further delineate the phylogeny of the Mycobacterium tuberculosis complex. Journal of bacteriology 188(12):4271-4287.

Jafarian, M., Aghali-Merza, M., Farnia, P., Ahmadi, M., Masjedi, M.R. and Velayati, A.A. (2010). Synchronous comparison of Mycobacterium tuberculosis epidemiology strains by “MIRU-VNTR” and “MIRU-VNTR and spoligotyping” technique. Avicenna journal of medical biotechnology 2(3):145.

Jagielski, T., Ignatowska, H., Bakuła, Z., Dziewit, Ł., Napiórkowska, A., Augustynowicz-Kopeć, E., Zwolska, Z. and Bielecki, J. (2014b). Screening for streptomycin resistance-conferring mutations in Mycobacterium tuberculosis clinical isolates from Poland. PloS One 9(6):e100078.

Jagielski, T., Minias, A., Van Ingen, J., Rastogi, N., Brzostek, A., Żaczek, A. and Dziadek, J. (2016). Methodological and clinical aspects of the molecular epidemiology of Mycobacterium tuberculosis and other mycobacteria. Clinical microbiology reviews 29(2):239-290.

Jagielski, T., Van Ingen, J., Rastogi, N., Dziadek, J., Mazur, P.K. and Bielecki, J. (2014). Current methods in the molecular typing of Mycobacterium tuberculosis and other mycobacteria. BioMed research international 2014.

Johansen, S.K., Maus, C.E., Plikaytis, B.B. and Douthwaite, S. (2006). Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2′-O-methylations in 16S and 23S rRNAs. Molecular cell, 23(2):173-182.

Jonsson, J., Hoffner, S., Berggren, I., Bruchfeld, J., Ghebremichael, S., Pennhag, A. and

Groenheit, R., 2014. Comparison between RFLP and MIRU-VNTR genotyping of Mycobacterium tuberculosis strains isolated in Stockholm 2009 to 2011. PloS One, 9(4):e95159.

Jugheli, L., Bzekalava, N., de Rijk, P., Fissette, K., Portaels, F. and Rigouts, L., (2009). High level of cross-resistance between kanamycin, amikacin, and capreomycin among Mycobacterium tuberculosis isolates from Georgia and a close relation with mutations in the rrs gene. Antimicrobial agents and chemotherapy, 53(12):5064-5068.

Juréen, P., Werngren, J., Toro, J.C. and Hoffner, S. (2008). Pyrazinamide resistance and pncA gene mutations in Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy 52(5):1852-1854.

Kafwabulula, M., Ahmed, K., Nagatake, T., Gotoh, J., Mitarai, S., Oizumi, K. and Zumla, A. (2002). Evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. The International Journal of Tuberculosis and Lung Disease 6(8):732-737.

Kamerbeek, J., Schouls, L.E.O., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M. and Van Embden, J. (1997). Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. Journal of clinical microbiology 35(4):907-914.

Karakousis, P. C. (2009). Mechanisms of action and resistance of antimycobacterial agents. In Antimicrobial drug resistance, pp. 271-291. Humana Press.

Karakousis, P.C., Bishai, W.R. and Dorman, S.E. (2004). Mycobacterium tuberculosis cell envelope lipids and the host immune response. Cellular microbiology 6(2):105-116.

Kariuki, S. (2010). Antimicrobial resistance in enteric pathogens in developing countries. In Antimicrobial Resistance in Developing Countries Springer, New York, pp. 177-197.

Kedir Abdella, Ketema Abdissa, Wakjira Kebede, Gemeda Abebe (2015). Drug resistance patterns of Mycobacterium tuberculosis complex and associated factors among retreatment cases around Jimma, Southwest Ethiopia. BMC public health 15(1):599.

Kelemework Adane, Gobena Ameni, Shiferaw Bekele, Markos Abebe and Abraham Aseffa (2015). Prevalence and drug resistance profile of Mycobacterium tuberculosis isolated from pulmonary tuberculosis patients attending two public hospitals in East Gojjam zone, northwest Ethiopia. BMC public health 15(1):572.

Kent, P.T. and Kubica, G.P. (1985). Public health mycobacteriology: a guide for the level III laboratory. US Department of Health and Human Services, Public Health Service, Centers for Disease Control.

Khan, S.N., Niemann, S., Gulfraz, M., Qayyum, M., Siddiqi, S., Mirza, Z.S., Tahsin, S., Ebrahimi-Rad, M. and Khanum, A. (2013). Molecular characterization of multidrug-resistant isolates of Mycobacterium tuberculosis from patients in Punjab, Pakistan. Pakistan J Zool. 45(1):93-100.

Kiepiela, P., Bishop, K.S., Smith, A.N., Roux, L. and York, D.F. (2000). Genomic mutations in the katG, inhA and aphC genes are useful for the prediction of isoniazid resistance in Mycobacterium tuberculosis isolates from Kwazulu Natal, South Africa. Tubercle and Lung Disease 80(1):47-56.

Koivula, T., Ekman, M., Leitner, T., Löfdahl, S., Ghebremicahel, S., Mostowy, S., Behr, M.A., Svenson, S.B. and Källenius, G. (2004). Genetic characterization of the Guinea-Bissau family of Mycobacterium tuberculosis complex strains. Microbes and infection 6(3):272-278.

Kolyva, A. S., and Karakousis, P. C. (2012). Old and new TB drugs: mechanisms of action and resistance. In Understanding Tuberculosis-New Approaches to Fighting Against Drug Resistance. InTech.

Kontsevaya, I.S., Nikolayevsky, V.V. and Balabanova, Y.M. (2011). Molecular epidemiology of tuberculosis: objectives, methods, and prospects. Molecular Genetics, Microbiology and Virology 26(1):1.

Kozhamkulov, U., Akhmetova, A., Rakhimova, S., Belova, E., Alenova, A., Bismilda, V., Chingissova, L., Ismailov, S., Ramanculov, E. and Momynaliev, K. (2011). Molecular Characterization of Rifampicin- and Isoniazid-Resistant Mycobacterium tuberculosis Strains Isolated in Kazakhstan. Japanese journal of infectious diseases, 64(3);253-255.

Kremer, K., Arnold, C., Cataldi, A., Gutiérrez, M.C., Haas, W.H., Panaiotov, S., Skuce, R.A., Supply, P., van der Zanden, A.G. and van Soolingen, D. (2005). Discriminatory power and reproducibility of novel DNA typing methods for Mycobacterium tuberculosis complex strains. Journal of clinical microbiology 43(11):5628-5638.

Kubica, G.P., Gross, W.M., Hawkins, J.E., Sommers, H.M., Vestal, A.L. and Wayne, L.G. (1975). Laboratory services for mycobacterial diseases. American Review of Respiratory Disease 112(6):773-787.

Lawn, S.D. and Wood, R. (2011). Tuberculosis in antiretroviral treatment services in resource-limited settings: addressing the challenges of screening and diagnosis. Journal of Infectious Diseases 204(4):S1159-S1167.

Lee, A.S., Lim, I.H., Tang, L.L. and Wong, S.Y. (2005). High frequency of mutations in the

rpoB gene in rifampin-resistant clinical isolates of Mycobacterium tuberculosis from Singapore. Journal of clinical microbiology 43(4):2026-2027.

Lee, H.Y., Myoung, H.J., Bang, H.E., Bai, G.H., Kim, S.J., Kim, J.D. and Cho, S.N. (2002). Mutations in the embB locus among Korean clinical isolates of Mycobacterium tuberculosis resistant to ethambutol. Yonsei Medical Journal 43(1):59-64.

Lee, K.W., Lee, J.M. and Jung, K.S. (2001). Characterization of pncA mutations of pyrazinamide-resistant Mycobacterium tuberculosis in Korea. Journal of Korean medical science 16(5):537.

Legesse Garedew, Adane Mihret, Gezahrgn Mamo, Tamrat Abebe,, Firdessa, R., Yonas Bekele, and Gobona Ameni (2013). Strain diversity of mycobacteria isolated from pulmonary tuberculosis patients at Debre Birhan Hospital, Ethiopia. The International Journal of Tuberculosis and Lung Disease 17(8):1076-1081.

Lemaitre, N., Sougakoff, W., Truffot-Pernot, C. and Jarlier, V. (1999). Characterization of new mutations in pyrazinamide-resistant strains of mycobacterium tuberculosisand identification of conserved regions important for the catalytic activity of the pyrazinamidase PncA. Antimicrobial agents and chemotherapy 43(7):1761-1763.

Liang, L., Wu, Q., Gao, L., Hao, Y., Liu, C., Xie, Y., Sun, H., Yan, X., Li, F., Li, H. and Fang, H. (2012). Factors contributing to the high prevalence of multidrug-resistant tuberculosis: a study from China. Thorax 2011. doi: 10.1136/thoraxjnl-2011-200018

Lienhardt, C., Vernon, A. and Raviglione, M.C. (2010). New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. Current opinion in pulmonary medicine 16(3):186-193.

Lipin, M.Y., Stepanshina, V.N., Shemyakin, I.G. and Shinnick, T.M. (2007). Association of specific mutations in katG, rpoB, rpsL and rrs genes with spoligotypes of multidrug-resistant Mycobacterium tuberculosis isolates in Russia. Clinical microbiology and infection 13(6):620-626.

Madison, B.M. (2001). Application of strains in clinical microbiology. Biotechnic and Histochemistry 76(3):119-125.

Mardassi, H., Namouchi, A., Haltiti, R., Zarrouk, M., Mhenni, B., Karboul, A., Khabouchi, N., van Pittius, N.C.G., Streicher, E.M., Rauzier, J. and Gicquel, B. (2005). Tuberculosis due to resistant Haarlem strain, Tunisia. Emerging infectious diseases 11(6):957.

Mathema, B., Kurepina, N.E., Bifani, P.J. and Kreiswirth, B.N. (2006). Molecular epidemiology of tuberculosis: current insights. Clinical microbiology reviews 19(4):658-685.

Maus, C.E., Plikaytis, B.B. and Shinnick, T.M. (2005). Mutation of tlyA confers capreomycin

resistance in Mycobacterium tubercu losis. Antimicrobial agents and chemotherapy 49(2):571-577.

Maus, C.E., Plikaytis, B.B. and Shinnick, T.M. (2005b). Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy 49(8):3192-3197.

Meseret Asefa and Meaza Demissie (2014). Risk Factors of Multi-Drug-resistant Tuberculosis in Addis Ababa, Ethiopia: A Matched Case–control Study. Open Access Library Journal, 1(3):1-8.

Migliori, G.B., Dheda, K., Centis, R., Mwaba, P., Bates, M., O'Grady, J., Hoelscher, M. and Zumla, A. (2010). Review of multidrug-resistant and extensively drug-resistant TB: global perspectives with a focus on sub-Saharan Africa. Tropical Medicine and International Health 15(9):1052-1066.

Migliori, G.B., Zellweger, J.P., Abubakar, I., Ibraim, E., Caminero, J.A., De Vries, G., D'Ambrosio, L., Centis, R., Sotgiu, G., Menegale, O. and Kliiman, K. (2012). European union standards for tuberculosis care.

Missirliu, A., Gasman, D., Vogt, B., Poveda, J.D., Abbou, C.C. and Chopin, D. (1996). Genitourinary tuberculosis: rapid diagnosis using the polymerase chain reaction. European urology, 30:523-524.

Mitchison, D.A. (1985). The action of antituberculosis drugs in short-course chemotherapy. Tubercle 66(3):219-225.

Mohajeri, P., Sadri, H., Farahani, A., Norozi, B. and Atashi, S. (2015). Frequency of mutations associated with rifampicin resistance in Mycobacterium tuberculosis strains isolated from patients in West of Iran. Microbial Drug Resistance 21(3):315-319.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J. and Mégraud, F. (1997). Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. Journal of clinical microbiology 35(4):995-998.

Moro, M.L., Gori, A., Errante, I., Infuso, A., Franzetti, F., Sodano, L., Iemoli, E. and Italian Multidrug-Resistant Tuberculosis Outbreak Study Group (1998). An outbreak of multidrug-resistant tuberculosis involving HIV-infected patients of two hospitals in Milan, Italy. Aids 12(9):1095-1102.

Mphahlele, M., Syre, H., Valvatne, H., Stavrum, R., Mannsåker, T., Muthivhi, T., Weyer, K., Fourie, P.B. and Grewal, H.M. (2008). Pyrazinamide resistance among South African multidrug-resistant Mycobacterium tuberculosis isolates. Journal of clinical microbiology 46(10):3459-3464.

Mulalem Agonafir, Eshetu Lemma, Dawit Wolde-Meskel, Solomon Goshu, Santhanam, A. Feven Girmachew, D Demissie, ; Getahun, M., Mekedes Gebeyehu, van Soolingen, D. (2010). Phenotypic and genotypic analysis of multidrug-resistant tuberculosis in Ethiopia. The International Journal of Tuberculosis and Lung Disease, 14(10):1259-1265.

Mulugeta Belay, Gobena Ameni, Gunnar Bjune, David Couvin, Nalin Rastogi, and Fekadu Abebe. (2014). Strain diversity of Mycobacterium tuberculosis isolates from pulmonary tuberculosis patients in Afar pastoral region of Ethiopia. BioMed research international, 2014.

Nasiri, M.J., Haeili, M., Ghazi, M., Goudarzi, H., Pormohammad, A., Imani Fooladi, A.A. and Feizabadi, M.M. (2017). New Insights in to the Intrinsic and Acquired Drug Resistance Mechanisms in Mycobacteria. Frontiers in microbiology 8:681.

Naveen, G. and PeeraPur, B.V. (2012). Comparison of the Lowenstein-Jensen medium, the Middlebrook 7H10 medium and MB/BacT for the isolation of Mycobacterium tuberculosis (MTB) from clinical specimens. Journal of Clinical and Diagnostic Research: 6(10):1704-1709.

Ndungu, P.W., Kariuki, S., Revathi, G. and Niemann, S. (2017). Mycobacteria Interspersed Repetitive Units-Variable Number of Tandem Repeat, Spoligotyping and Drug Resistance of Isolates from Pulmonary Tuberculosois Patients in Kenya. Advances in Microbiology 7(03):205.

Niemann, S., Rüsch-Gerdes, S., Richter, E., Thielen, H., Heykes-Uden, H. and Diel, R. (2000). Stability of IS6110 Restriction Fragment Length Polymorphism Patterns of Mycobacterium tuberculosisStrains in Actual Chains of Transmission. Journal of clinical microbiology 38(7):2563-2567.

Oelemann, M.C., Diel, R., Vatin, V., Haas, W., Rüsch-Gerdes, S., Locht, C., Niemann, S. and Supply, P. (2007). Assessment of an optimized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. Journal of clinical microbiology 45(3):691-697.

Oelemann, M.C., Gomes, H.M., Willery, E., Possuelo, L., Lima, K.V.B., Allix-Béguec, C.,

Locht, C., Gutierrez, M.C., Suffys, P. and Supply, P. (2011). The forest behind the tree: phylogenetic exploration of a dominant Mycobacterium tuberculosis strain lineage from a high tuberculosis burden country. PloS One 6(3):e18256.

Okamoto, S., Tamaru, A., Nakajima, C., Nishimura, K., Tanaka, Y., Tokuyama, S., Suzuki, Y.

and Ochi, K. (2007). Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Molecular microbiology 63(4):1096-1106.

Ong, D.C., Yam, W.C., Siu, G.K. and Lee, A.S. (2010). Rapid detection of rifampicin-and isoniazid-resistant Mycobacterium tuberculosis by high-resolution melting analysis. Journal of clinical microbiology 48(4):1047-1054.

Palomino, J.C. and Martin, A. (2014). Drug resistance mechanisms in Mycobacterium tuberculosis. Antibiotics 3(3):317-340.

Paranjothy, S., Eisenhutt, M., Lilley, M.E., Bracebridge, S., Abubakar, I., Mulla, R., Lack, K., Chalkley, D., Howard, J., Thomas, S. and McEvoy, M. (2008). Extensive Transmission of Mycobacterium Tuberculosis from a 9-Year Old Child with Sputum-Smear-Negative Pulmonary Tuberculosis. International Journal of Infectious Diseases 12:e49.

Parsons, L.M., Somoskövi, Á., Gutierrez, C., Lee, E., Paramasivan, C.N., Abimiku, A.L., Spector, S., Roscigno, G. and Nkengasong, J. (2011). Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clinical microbiology reviews 24(2):314-350.

Peter, J.G., Theron, G., Muchinga, T.E., Govender, U. and Dheda, K. (2012). The diagnostic accuracy of urine-based Xpert MTB/RIF in HIV-infected hospitalized patients who are smear-negative or sputum scarce. PloS One 7(7):e39966.

Pfyffer, G.E. and Palicova, F. (2011). Mycobacterium: general characteristics, laboratory detection, and staining procedures. In Manual of Clinical Microbiology, 10th Edition. American Society of Microbiology, pp. 472-502.

Piatek, A.S., Telenti, A., Murray, M.R., El-Hajj, H., Jacobs, W.R., Kramer, F.R. and Alland, D. (2000). Genotypic analysis of Mycobacterium tuberculosis in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrobial agents and chemotherapy 44(1):103-110.

Porth, C.M. (2002). Alterations in respiratory function: respiratory tract infections, neoplasms, and childhood disorders. Porth CM, Kunert MP. Pathophysiology: Concepts of Altered Health States. Philadelphia, PA: Lippincott Williams and Wilkins, pp. 615-619.

Prammananan, T., Cheunoy, W., Taechamahapun, D., Yorsangsukkamol, J., Phunpruch, S., Phdarat, P., Leechawengwong, M. and Chaiprasert, A. (2008). Distribution of rpoB mutations among multidrug-resistant Mycobacterium tuberculosis (MDRTB) strains from Thailand and development of a rapid method for mutation detection. Clinical Microbiology and Infection 14(5):446-453.

Prosser, G.A. and de Carvalho, L.P. (2013). Metabolomics reveal d-alanine: d-alanine ligase as the target of d-cycloserine in Mycobacterium tuberculosis. ACS medicinal chemistry letters 4(12):1233-1237.

Qazi, O., Rahman, H., Tahir, Z., Qasim, M., Khan, S., Anjum, A.A., Yaqub, T., Tayyab, M., Ali, N. and Firyal, S. (2014). Mutation pattern in rifampicin resistance determining region of rpoB gene in multidrug-resistant Mycobacterium tuberculosis isolates from Pakistan. International journal of mycobacteriology 3(3):173-177.

Racheal, S., Zephaniah, D., Reggie, M., Kerina, D., Babill, S.P. and Peter, M. (2015). Diagnosis of Multi-drug-resistant Tuberculosis Mutations Using Hain Line Probe Assay and GeneXpert" A Study Done in Zimbabwe. British Journal of Medicine and Medical Research 5(8):1044-1052.

Ramaswamy, S. and Musser, J.M. (1998). Molecular genetic basis of antimicrobial agent resistance inMycobacterium tuberculosis: 1998 update. Tubercle and Lung disease 79(1):3-29.

Ramaswamy, S.V., Amin, A.G., Göksel, S., Stager, C.E., Dou, S.J., El Sahly, H., Moghazeh, S.L., Kreiswirth, B.N. and Musser, J.M. (2000). Molecular Genetic Analysis of Nucleotide Polymorphisms Associated with Ethambutol Resistance in Human Isolates of Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy 44(2):326-336.

Rattan, A., Kalia, A. and Ahmad, N. (1998). Multidrug-resistant Mycobacterium tuberculosis: molecular perspectives. Emerging infectious diseases, 4(2):195-209.

Rebollo, M.J., Garrido, R.S.J., Folgueira, D., Palenque, E., Diaz-Pedroche, C., Lumbreras, C. and Aguado, J.M. (2006). Blood and urine samples as useful sources for the direct detection of tuberculosis by polymerase chain reaction. Diagnostic microbiology and infectious disease 56(2):141-146.

Reid, M.J. and Shah, N.S. (2009). Approaches to tuberculosis screening and diagnosis in people with HIV in resource-limited settings. The Lancet infectious diseases, 9(3):173-184.

Rieder, H.L. (1999). Epidemiologic basis of tuberculosis control. Ed. 1. International Union Against Tuberculosis and Lung Disease (IUATLD):1-162

Riley, R.L., Mills, C.C., Nyka, W., Weinstock, N., Storey, P.B., Sultan, L.U., Riley, M.C. and Wells, W.F. (1959). Aerial dissemination of pulmonary tuberculosis. A two-year study of contagion in a tuberculosis ward. American Journal of Hygiene 70(2):185-96.

Rozwarski, D.A., Grant, G.A., Barton, D.H., Jacobs, W.R. and Sacchettini, J.C. (1998).

Modification of the NADH of the isoniazid target (InhA) from Mycobacterium tuberculosis. Science 279(5347):98-102.

Safi, H., Sayers, B., Hazbón, M.H. and Alland, D. (2008). Transfer of embB codon 306 mutations into clinical Mycobacterium tuberculosis strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrobial agents and chemotherapy 52(6):2027-2034.

Salfinger, M., Crowle, A.J. and Reller, L.B. (1990). Pyrazinamide and pyrazinoic acid activity against tubercle bacilli in cultured human macrophages and in the BACTEC system. Journal of Infectious Diseases 162(1):201-207.

Sandgren, A., Strong, M., Muthukrishnan, P., Weiner, B.K., Church, G.M. and Murray, M.B. (2009). Tuberculosis drug resistance mutation database. PLoS medicine 6(2):e1000002.

Scorpio, A. and Zhang, Y. (1996). Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nature medicine 2(6):662-667.

Selamawit Hirpa, Girmay Medhin, Belaineh Girma, Muluken Melese, Alemayehu Mekonen, Pedro Suarez, Gobena Ameni (2013). Determinants of multidrug-resistant tuberculosis in patients who underwent first-line treatment in Addis Ababa: a case control study. BMC Public Health 13(1):782.

Sharma, S.K. and Mohan, A. (2004). Multidrug-resistant tuberculosis. Indian Journal of Medical Research 120:354-376.

Sharma, S.K., Kumar, S., Saha, P.K., George, N., Arora, S.K., Gupta, D., Singh, U., Hanif, M. and Vashisht, R.P. (2011). Prevalence of multidrug-resistant tuberculosis among category II pulmonary tuberculosis patients.

Sheen, P., Ferrer, P., Gilman, R.H., López-Llano, J., Fuentes, P., Valencia, E. and Zimic, M.J. (2009). Effect of pyrazinamidase activity on pyrazinamide resistance in Mycobacterium tuberculosis. Tuberculosis 89(2):109-113.

Shi, W., Chen, J., Feng, J., Cui, P., Zhang, S., Weng, X., Zhang, W. and Zhang, Y. (2014). Aspartate decarboxylase (PanD) as a new target of pyrazinamide in Mycobacterium tuberculosis. Emerging microbes and infections 3(8):e58.

Shou-Yean Grace Lin, MS, Edward P. Desmond. (2014). Molecular diagnosis of tuberculosis and drug resistance. Clinics in laboratory medicine 34(2):297-314.

Shubladze, N., Tadumadze, N. and Bablishvili, N. (2013). Molecular patterns of multidrug resistance of Mycobacterium tuberculosis in Georgia. International journal of mycobacteriology 2(2):73-78.

Siddiqi, S.H. and Rüsch-Gerdes, S. (2006). MGIT procedure manual. Geneva, Switzerland: Foundation for innovative new diagnostics, pp.41-51.

Smittipat, N., Billamas, P., Palittapongarnpim, M., Thong-On, A., Temu, M.M., Thanakijcharoen, P., Karnkawinpong, O. and Palittapongarnpim, P. (2005). Polymorphism of variable-number tandem repeats at multiple loci in Mycobacterium tuberculosis. Journal of clinical microbiology 43(10):5034-5043.

Solomon Abebe Yimer. Gunnstein Norheim, Amine Namouchi, Ephrem Debebe Zegeye, Wibeke Kinander, Tone Tønjum, Shiferaw Bekele, Turid Mannsåker, Gunnar Bjune, Abraham Aseffa, Carol Holm-Hansen (2015). Mycobacterium tuberculosis lineage 7 strains are associated with prolonged patient delay in seeking treatment for pulmonary tuberculosis in Amhara Region, Ethiopia. Journal of clinical microbiology 53(4):1301-1309.

Somerville, W., Thibert, L., Schwartzman, K. and Behr, M.A. (2005). Extraction of Mycobacterium tuberculosis DNA: a question of containment. Journal of clinical microbiology 43(6):2996-2997.

Spies, F.S., Da Silva, P.E.A., Ribeiro, M.O., Rossetti, M.L. and Zaha, A. (2008). Identification of mutations related to streptomycin resistance in clinical isolates of Mycobacterium tuberculosis and possible involvement of efflux mechanism. Antimicrobial agents and chemotherapy 52(8):2947-2949.

Spies, F.S., Ribeiro, A.W., Ramos, D.F., Ribeiro, M.O., Martin, A., Palomino, J.C., Rossetti, M.L.R., da Silva, P.E.A. and Zaha, A. (2011). Streptomycin resistance and lineage-specific polymorphisms in Mycobacterium tuberculosis gidB gene. Journal of clinical microbiology 49(7):2625-2630.

Sreevatsan, S., Pan, X., Zhang, Y., Kreiswirth, B.N. and Musser, J.M. (1997a). Mutations associated with pyrazinamide resistance in pncA of Mycobacterium tuberculosis complex organisms. Antimicrobial agents and chemotherapy 41(3):636-640.

Sreevatsan, S., Stockbauer, K.E., Pan, X.I., Kreiswirth, B.N., Moghazeh, S.L., Jacobs, W.R., Telenti, A. and Musser, J.M. (1997b). Ethambutol resistance in Mycobacterium tuberculosis: critical role of embB mutations. Antimicrobial Agents and Chemotherapy 41(8):1677-1681.

Stavrum, R., Myneedu, V.P., Arora, V.K., Ahmed, N. and Grewal, H.M. (2009). In-depth molecular characterization of Mycobacterium tuberculosis from New Delhi–predominance of drug-resistant isolates of the 'Modern' (TbD1−) type. PloS One 4(2):e4540.

Sun, Y.J., Luo, J.T., Wong, S.Y. and Lee, A.S.G. (2010). Analysis of rpsL and rrs mutations

in Beijing and non-Beijing streptomycin-resistant Mycobacterium tuberculosis isolates from Singapore. Clinical Microbiology and Infection 16(3):287-289.

Sun, Z., Zhang, J., Zhang, X., Wang, S., Zhang, Y. and Li, C. (2008). Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant Mycobacterium tuberculosis strains and clinical isolates. International journal of antimicrobial agents, 31(2):115-121.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S. and Bifani, P. (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. Journal of clinical microbiology 44(12):4498-4510.

Supply, P., Lesjean, S., Savine, E., Kremer, K., Van Soolingen, D. and Locht, C. (2001). Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. Journal of clinical microbiology 39(10):3563-3571.

Supply, P., Magdalena, J., Himpens, S. and Locht, C. (1997). Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Molecular microbiology 26(5):991-1003.

Suzuki, Y., Katsukawa, C., Tamaru, A., Abe, C., Makino, M., Mizuguchi, Y. and Taniguchi, H. (1998). Detection of Kanamycin-ResistantMycobacterium tuberculosis by Identifying Mutations in the 16S rRNA Gene. Journal of clinical microbiology 36(5):1220-1225.

Takiff, H.E., Salazar, L., Guerrero, C., Philipp, W., Huang, W.M., Kreiswirth, B., Cole, S.T., Jacobs, W.R. and Telenti, A. (1994). Cloning and nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrobial agents and chemotherapy 38(4):773-780.

Tan, Y., Hu, Z., Zhang, T., Cai, X., Kuang, H., Liu, Y., Chen, J., Yang, F., Zhang, K., Tan, S. and Zhao, Y. (2014). Role of pncA and rpsA gene sequencing in detection of pyrazinamide resistance in Mycobacterium tuberculosis isolates from southern China. Journal of clinical microbiology 52(1):291-297.

Tanveer, M., Hasan, Z., Siddiqui, A.R., Ali, A., Kanji, A., Ghebremicheal, S. and Hasan, R. (2008). Genotyping and drug resistance patterns of M. tuberculosis strains in Pakistan. BMC infectious diseases 8(1):171.

Tekwu, E.M., Sidze, L.K., Assam, J.P.A., Tedom, J.C., Tchatchouang, S., Makafe, G.G.,

Wetewale, A.L.T., Kuaban, C., Eyangoh, S., Ntoumi, F. and Beng, V.N.P. (2014). Sequence analysis for detection of drug resistance in Mycobacterium tuberculosis complex isolates from the Central Region of Cameroon. BMC microbiology 14(1):113.

Telenti, A., Honore, N., Bernasconi, C.A., March, J., Ortega, A., Heym, B., Takiff, H.E. and Cole, S.T. (1997). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at reference laboratory level. Journal of clinical microbiology 35(3):719-723.

Telenti, A., Philipp, W.J., Sreevatsan, S., Bernasconi, C., Stockbauer, K.E., Wieles, B., Musser, J.M. and Jacobs Jr, W.R. (1997b). The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nature medicine 3(5):567-570.

Theron, G., Peter, J., van Zyl-Smit, R., Mishra, H., Streicher, E., Murray, S., Dawson, R., Whitelaw, A., Hoelscher, M., Sharma, S. and Pai, M. (2011). Evaluation of the Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in a high HIV prevalence setting. American journal of respiratory and critical care medicine 184(1):132-140.

Thong-On, A., Smittipat, N., Juthayothin, T., Yanai, H., Yamada, N., Yorsangsukkamol, J., Chaiprasert, A., Rienthong, D., Billamas, P. and Palittapongarnpim, P. (2010). Variable-number tandem repeats typing of Mycobacterium tuberculosis isolates with low copy numbers of IS6110 in Thailand. Tuberculosis 90(1):9-15.

Torrea, G., Van de Perre, P., Ouedraogo, M., Zougba, A., Sawadogo, A., Dingtoumda, B., Diallo, B., Defer, M.C., Sombie, I., Zanetti, S. and Sechi, L.A. (2005). PCR-based detection of the Mycobacterium tuberculosis complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso. Journal of medical microbiology, 54(1), pp.39-44.

Tostmann, A., Kik, S.V., Kalisvaart, N.A., Sebek, M.M., Verver, S., Boeree, M.J. and van Soolingen, D. (2008). Tuberculosis transmission by patients with smear-negative pulmonary tuberculosis in a large cohort in the Netherlands. Clinical Infectious Diseases 47(9):1135-1142.

Van Embden, J.D.A., Van Gorkom, T., Kremer, K., Jansen, R., Van der Zeijst, B.A.M. and Schouls, L.M. (2000). Genetic variation and evolutionary origin of the direct repeat locus of Mycobacterium tuberculosis complex bacteria. Journal of bacteriology 182(9):2393-2401.

Van Hung, N., Sy, D.N., Anthony, R.M., Cobelens, F.G. and van Soolingen, D. (2007).

Fluorescence microscopy for tuberculosis diagnosis. The Lancet infectious diseases 7(4):238-239.

van Ingen, J. (2013). Diagnosis of nontuberculous mycobacterial infections. In Seminars in respiratory and critical care medicine 34 (1):103-109. Thieme Medical Publishers.

Van Rie, A., Victor, T.C., Richardson, M., Johnson, R., Van Der Spuy, G.D., Murray, E.J., Beyers, N., Van Pittius, N.C.G., Van Helden, P.D. and Warren, R.M. (2005). Reinfection and mixed infection cause changing Mycobacterium tuberculosis drug-resistance patterns. American journal of respiratory and critical care medicine 172(5):636-642.

Van Soolingen, D. (2001). Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of internal medicine 249(1):1-26.

Varghese, B., Shoukri, M., Memish, Z., Abuljadayel, N., Alhakeem, R., Alrabiah, F. and Al-Hajoj, S. (2014). Occurrence of diverse mutations in isoniazid-and rifampicin-resistant Mycobacterium tuberculosis isolates from autochthonous and immigrant populations of Saudi Arabia. Microbial drug resistance 20(6):623-631.

Victor, T.C., Streicher, E.M., Kewley, C., Jordaan, A.M., Van Der Spuy, G.D., Bosman, M., Louw, H., Murray, M., Young, D., Van Helden, P.D. and Warren, R.M. (2007). Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the western Cape of South Africa. The International Journal of Tuberculosis and Lung Disease 11(2):195-201.

Vilcheze, C., Weisbrod, T.R., Chen, B., Kremer, L., Hazbón, M.H., Wang, F., Alland, D., Sacchettini, J.C. and Jacobs, W.R. (2005). Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. Antimicrobial Agents and Chemotherapy 49(2):708-720.

Wagner, D. and Young, L.S. (2004). Nontuberculous mycobacterial infections: a clinical review. Infection 32(5):257-270.

Wall, S., Ghanekar, K., McFadden, J. and Dale, J.W. (1999). Context-sensitive transposition of IS6110 in mycobacteria. Microbiology 145(11):3169-3176.

Weiss, C.H. and Glassroth, J. (2012). Pulmonary disease caused by nontuberculous mycobacteria. Expert Rev Respir Med. 6:597–613.

Wiedenheft, B., Sternberg, S.H. and Doudna, J.A., 2012. RNA-guided genetic silencing systems in bacteria and archaea. Nature 482(7385):331-338.

Wondemagegn Mulu, Bayeh Abera, Mulat Yimer, Tadesse Hailu, Haimanot Ayele, Dereje

Abate (2017). Rifampicin-resistance pattern of Mycobacterium tuberculosis and associated factors among presumptive tuberculosis patients referred to Debre Markos Referral Hospital, Ethiopia: a cross-sectional study. BMC research notes 10(1):8.

Woods, G.L. (2011). Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. Approved Standard M24-A2, 31(5).

WHO (1998). Laboratory Service in Tuberculosis Control: Part II. Microscopy, World Health Organization, Geneva, Switzerland.

WHO (2005). Tuberculosis. Fact sheet No 104 Revised. World Health Organization, Geneva, Switzerland. Available at http://www.who.int/mediacentre/factsheets/fs104/en/index.html.

WHO (2008). Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): policy statement [Internet]. Geneva: World Health Organization, Geneva, Switzerland. Available at: http://www.who.int/tb/laboratory/lpa_policy.pdf. Accessed on 15 July, 2015.

WHO (2010). Multidrug and extensively drug-resistant TB (M/XDR-TB): Global report on surveillance and response. World Health Organization. Geneva, Switzerland. Available at: http://www.who.int/tb/publications/global_report/en/ Accessed October 20, 2015.

WHO (2011a). Rapid Implementation of the Xpert MTB/ RIF diagnostic test. Technical and operational 'How-to' Practical considerations. World Health Organization, Geneva, Switzerland. Available at: http://www.who.int/tb/publications/global_report/en/

WHO (2011b). Towards universal access to diagnosis and treatment of multidrug-resistant and extensively drug-resistant tuberculosis by 2015: WHO progress report 2011. Geneva, Switzerland.

WHO (2012). Updated interim critical concentrations for first-line and second-line DST. World Health Organization, Geneva, Switzerland. http://www.stoptb.org/wg/gli/assets/documents/ Accessed on 15 July, 2015.

WHO (2013a). Global tuberculosis report 2013. World Health Organization.

WHO (2013b). Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MT. Accessed June 20, 2014 at http://www.who.int/tb/laboratory/policy_statements/en/.

WHO (2015). WHO Ethiopia UPDATE sheet on TB programme in 2014. World Health Organization. Accessed 20 May 2015 at <www.afro.who.int/en/ethiopia/who-country-office-ethiopia.html>

WHO (Ed.) (2013). Global tuberculosis report 2013. World Health Organization. Geneva,

Switzerland. Available at: http://wh1libdoc.who.int/publications/2013/9789241564656_eng.pdf. .

WHO (Ed.). (2016). Global tuberculosis report 2016. World Health Organization. http://www.who.int/tb/publications/global_report/en/ Accessed February 14, 2017.

WHO (Ed.). (2017). Global tuberculosis report 2017. World Health Organization. http://www.who.int/tb/publications/global_report/en/ Accessed October 25, 2017.

WHO and Stop TB Initiative (World Health Organization) (2010b). Treatment of tuberculosis: guidelines. 4th edition. World Health Organization. Geneva, Switzerland.

Yadav, R., Sethi, S., Dhatwalia, S.K., Gupta, D., Mewara, A. and Sharma, M. (2013). Molecular characterisation of drug resistance in Mycobacterium tuberculosis isolates from North India. The International Journal of Tuberculosis and Lung Disease 17(2):251-257.

Yohanes Abate, Solomon Abera, and Solomon Ali (2012). Smear positive pulmonary tuberculosis among suspected patients attending metehara sugar factory hospital; eastern Ethiopia. African health sciences, 12(3), pp.325-330.

Zhang, Y. and Telenti, A. (2000). Genetics of drug resistance in Mycobacterium tuberculosis Molecular genetics of mycobacteria. ASM Press, Washington, DC, pp. 235-254.

Zhang, Y. and Yew, W.W. (2009). Mechanisms of drug resistance in Mycobacterium tuberculosis [State of the art series. Drug-resistant tuberculosis. Edited by CY. Chiang. Number 1 in the series]. The International Journal of Tuberculosis and Lung Disease 13(11):1320-1330.

Zhang, Y. and Yew, W.W. (2015). Mechanisms of drug resistance in Mycobacterium tuberculosis: update 2015. The International Journal of Tuberculosis and Lung Disease 19(11):1276-1289.

Zhang, Y., Heym, B., Allen, B., Young, D. and Cole, S. (1992). The catalase—peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 358(6387):591.

Zhao, J.R., Bai, Y.J., Wang, Y., Zhang, Q.H., Luo, M. and Yan, X.J. (2005). Development of a pyrosequencing approach for rapid screening of rifampin, isoniazid and ethambutol-resistant Mycobacterium tuberculosis. The International Journal of Tuberculosis and Lung Disease 9(3):328-332.

Zhao, L.L., Sun, Q., Liu, H.C., Wu, X.C., Xiao, T.Y., Zhao, X.Q., Li, G.L., Jiang, Y., Zeng, C.Y. and Wan, K.L. (2015). Analysis of embCAB mutations associated with ethambutol resistance in multidrug-resistant Mycobacterium tuberculosis isolates from China. Antimicrobial agents and chemotherapy 59(4):2045-2050

Annexes

Annex I: Information Sheet

Information Sheet for Data Collection on Drug-Resistant TB and Risk Factors for Development of Drug-Resistant TB and and evalution of the Performance of GeneXpert Assay Using Urine for Diagnosis of Pulmonary TB

Addis Ababa University College of Natural Science Department of Microbial, Cellular and Molecular Biology

Information Sheet

Identification: Health Facility Name_____ Phone _____
Health Facility Address: Sub city_____ Woreda_____
Clint Code *(this code should be also given to specimen)*_____

Dear participant,

My name is _____, I am working as data collector in a study conducted by the Addis Ababa University College of Natural Science Department of Microbial, Cellular and Molecular Biology. I would like to interview you few questions about Drug-Resistant TB and Risk factors for development of drug-resistant TB. The objective of the study is to identify risk factors for development of drug-resistant TB and to generate knowledge that contributes for the improvement of diagnosis, treatment and prevention of MDR-TB program in Ethiopia. You are kindly requested to be included in the study, which will have importance in improving TB control program and your cooperation and willingness for interview and providing specimens will be very helpful in identifying the problems related to the issue. Specimen that you provide will be sputum and or urine and there is no any invasive procedure applying or we can use your left over specimens if available. You can give these specimens within few minutes without any precondition and invasive procedure and giving of these specimens does not affect your health. Your name will not be written in the form and specimen, I assure you all the information and specimens you give will be kept strictly confidential. Your participation is voluntary and you are not obliged to answer any questions and give any specimens that you do not want to answer. I would appreciate if you would take few moment of your valuable time to answer this questionnaire and give specimens. Thank you in advance in anticipation of your cooperation. If you are not comfortable with the interview, please feel free to stop it any time you like. Do I have your permission to continue? If yes, continue to next page for the interview. If no, continue to next participant

I thank you for your cooperation

Address: Investigator: Eyob Abera*: Tel +251911637525 Email:eyob2001@gmail.com*

Department of Microbial, Cellular and Molecular Biology, Addis Ababa University

Interviewer's Signature _____ Date of interview _____
Supervisor's Name _____ Signature_____ Date_____

Annex II: Consent Form

Consent Form for Data Collection on Drug-Resistant TB and Risk Factors for Development of Drug-Resistant TB and evalution of the Performance of GeneXpert Assay Using Urine for Diagnosis of Pulmonary TB

Addis Ababa University College of Natural Science Department of Microbial, Cellular and Molecular Biology

Consent Form

I have read the information sheet above and clearly understood the purpose and anticipated benefit of the research. I hereby need to assure with my signature below that I, without any coercion or forceful act by the research team, have decided to voluntarily participate in the study to give necessarily information and specimens (sputum and urine) to contribute my part in the effort being made for the betterment of TB control and prevention program in Ethiopia.

Participant Code_____ Signature_____ Date_____

Interviewer's Name _____ Signature_____ Date _____

Annex III: Questionnaire for Data Collection on Drug-Resistant TB

Questionnaire for Data Collection on Drug-Resistant TB, and Risk Factors for Development of Drug-Resistant TB

Addis Ababa University College of Natural Science Department of Microbial, Cellular and Molecular Biology

General direction:

Please put a number in the column of "**code**" for the answer. In cases where the responses other than mark are required please write your response in the space provided and also more than one answer is possible. This Questionnaire has 4 sections and data collector should fill section I-III accordingly and **section IV** will be filled after sample analysis. **Note:** data for section III will be found from TB Register.

SECTION I: SOCIO-DEMOGRAPHIC AND ECONOMIC INFORMATION				
S.No	**Questions**	**Responses**	**Code**	**Remark**
1.	Sex	1. Male 2. Female		
2.	Your age	1. Year_____ 2. No response		
3.	Living Address (Region)	Region_____		
4.	Living Place	1. Urban 2. Rural 3. Other		
5.	What is your religion?	1. Orthodox 2. Muslim 3. Protestant 4. Catholic 5. Other (specify)		
6.	What is your ethnic group	1. Amhara 2. Oromo 3. Tigre 4. Gurage 5. Didn't mention 6. Other _____		
7.	What is your current marital status?	1. Married 2. Divorced 3. Widowed 4. Unmarried		
8.	What is your completed educational status?	1 Illiterate 2 Read and write 3 Elementary school Completed 4 High school Completed 5 College Graduate 6 Others (specify) _____		
9.	What is your current occupation?	1. Civil servant 2. House wife 3. Daily laborer 4. Student 5. Self employed 6. Health professionals 7. Others(specify)		
10.	What is your average household income per month?	1. _____ birr 2. No income 3. Others(specify)		

11.	What is your number of rooms in home?	1. _____room/s 2. No response		
12.	What is your Family size	1. _____ 2. No response		
SECTION II: TB and Risk Factors for Development Drug resistance				
13.	Do you have any family member TB patient now and/or before?	1 Yes 2 No 3 No response Yes		
14.	If yes for the above question, did he/she take the medication	1 Yes 2 No 3 No response Yes		
15.	Have you ever been being infected with TB before?	1 Yes 2 No 3 No response		
16.	If yes for the above question, have you ever taken TB Drug?	1 Yes 2 No 3 No response		
17.	If yes for the above question, have you ever been dis-continued the drug of TB?	1. Yes 2. No 3. No response		
18.	If yes for the above question, for how long did you discontinue the medication	1. _____days 2. I don't remember 3. No response		
19.	What was the reason for interruption for the drug?	1 Side effects 2 Forgetting 3 Symptoms were gone and felt good 4 Shortage of drug		
20.	How many times did you discontinue the medication?	1. _____ times 2. I don't remember 3. No response		
21.	Did you continue your medication, after discontinuing the drug of TB?	1. Yes 2. No 3. No response		
22.	Have you ever faced any drug side effects during treatment of TB?	1 Yes 2 No 3 No response		
23.	How long did you take the drug for the first-time TB treatment	1. _____ days 2. I don't remember 3. No response		
24.	Has the TB infection ever been relapsed after your treatment?	1 Yes 2 No 3 No response		
25.	Have you ever been directly observed by health worker while taking anti-TB	1. Yes 2. No 3. No response		
26.	Have you ever switched anti-TB drug?	1. Yes 2. No 3. No response		
27.	Have you ever taken any antibiotic drug frequently?	1 Yes 2 No 3 No response		
28.	If yes for the above question, have you ever interrupted the antibiotic?	1 Yes 2 No 3 No response		

29.	How many times have you interrupted the antibiotic?	1. _____ times 2. I don't remember 3. No response		
30.	Have you ever taken alcohol regularly?	1 Yes 2 No 3 No response		
31.	Have you ever taken alcohol during the treatment?	1 Yes 2 No 3 No response		
32.	Have you ever been smoking regularly?	1 Yes 2 No 3 No response		
33.	Have you ever been tested for HIV?	1 Yes 2 No 3 No response		
34.	If yes for HIV testing, how was Your result?	1 Positive 2 Negative 3 Don't want to disclose		
35.	Have you ever been admitted to hospital?	1. Yes 2. No 3. No response		
36.	If yes for the above question,, how long have you ever stayed in hospital?	1. _____ days 2. I don't remember 3. No response		
37.	Have you ever visited frequently health facility before?	1. Many times 2. Sometimes 3. Never visit 4. No response		
SECTION III: TB and Treatment History from TB register				
38.	Treatment History?	1 New 2 Retreatment		
39.	TB history	1 News 2 Relapse 3 Defaulted 4 Treatment failure 5 Other 6 Unknown		
40.	Drug regimen (category) for the second time	1 Category I 2 Category II		
SECTION IV: Laboratory Results(To be filled after sample analysis)				
A.	AFB/ZN Microscope:			
B.	GeneXpert			
C.	Culture			
D.	Drug Sensitivity Test • RIF:_____ • INH: _____ • STR: _____ • ETB: _____ • PYR: _____			

----------END----------

Thank you for your participation

Annex IV: Questionnaire for Data Collection for Evalution of GneXpert

Questionnaire for Data Collection on Evalution of performance of GneXpert Assay using Urine as alternative Specimen.

General direction:

Please put a number in the column of "**code**" for the answer. In cases where the responses other than mark are required please write your response in the space provided and also more than one answer is possible. This Questionnaire has 4 sections and data collector should fill section I-III accordingly. **Note:** data for section III will be found from TB Register.

SECTION I: SOCIO-DEMOGRAPHIC AND ECONOMIC INFORMATION				
S.No	**Questions**	**Responses**	**Code**	**Remark**
41.	Sex	1. Male 2. Female		
3.	Your age	1. Year_____ 2. No response		
3.	Living Address (Region)	Region_____		
4.	Living Place	1. Urban 2. Rural		
5.	What is your current marital status?	1. Married 2. Divorced 3. Widowed 4. Unmarried		
6.	What is your current occupation?	1. Civil servant 2. House wife 3. Daily laborer 4. Student 5. Self employed 6. Health professionals 7. Others(specify)		
SECTION II: TB History				
7.	Do you have sputum now?	1. Yes 2. No 3. No response		
8.	Have you ever been infected with TB before? ***If "No" go to Question 13***	1. Yes 2. No 3. No response		
9.	If yes to the above question, have you ever taken TB Drug?	1. Yes 2. No 3. No response		
10.	If yes to the above question, have you ever been dis-continued the drug of TB?	1. Yes 2. No 3. No response		
11.	If yes for the above question, did you continue your medication?	1. Yes 2. No 3. No response		
12.	Has the TB infection ever been relapsed after your treatment?	1. Yes 2. No 3. No response		
13.	Have you taken alcohol regularly?	1. Yes 2. No 3. No response		

14.	Have you been smoking regularly?	1. Yes 2. No 3. No response		
15.	Have you ever been tested for HIV?	1. Yes 2. No 3. No response		
16.	If yes for HIV testing, what was your result?	1. Positive 2. Negative 3. Don't want to disclose		

SECTION III: Laboratory Results(To be filled after sample analysis)

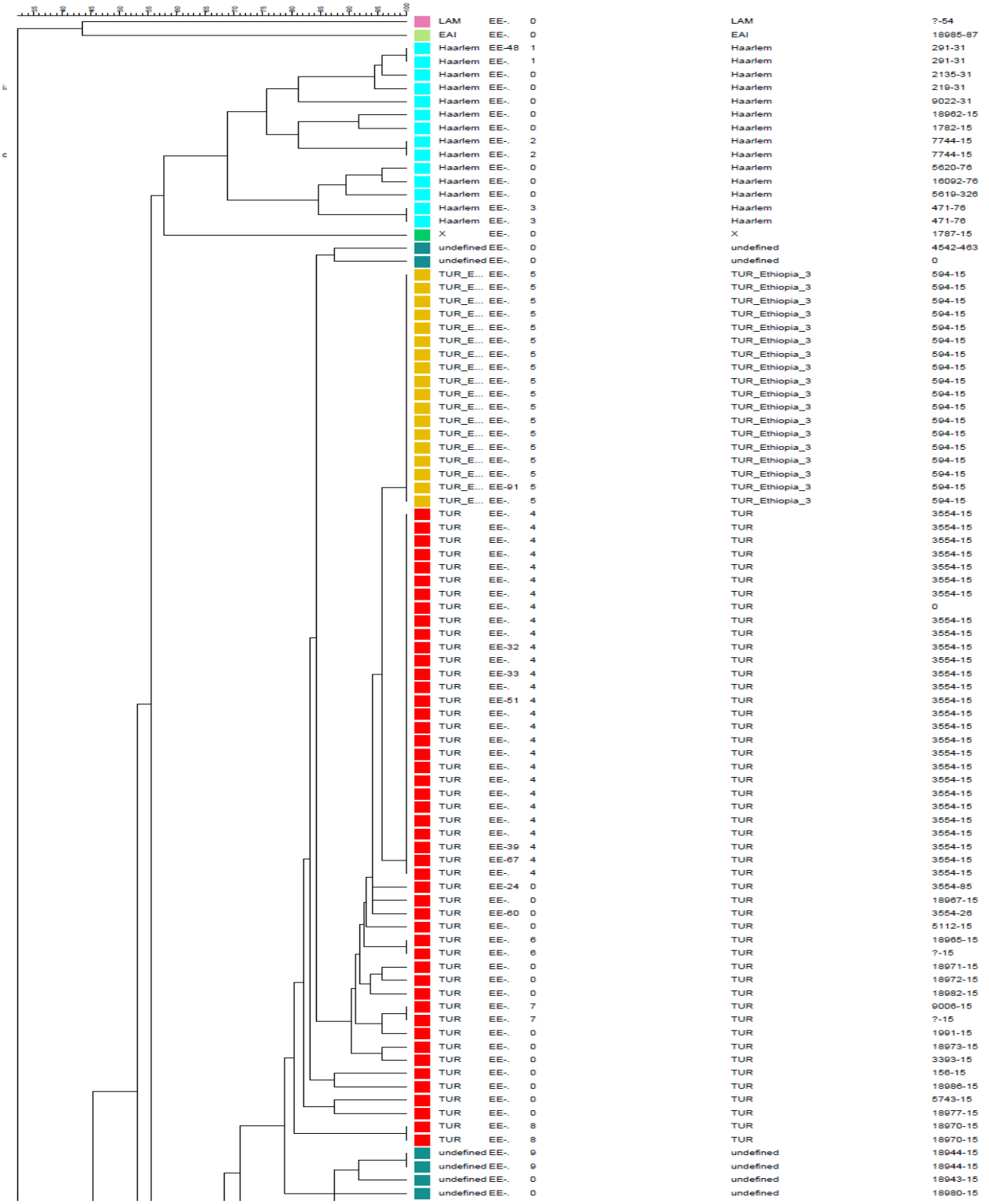
	Test Method	Urine Specimen	Sputum
17.	AFB/ZN Microscope:		
18.	GeneXpert		
19.	Culture		
20.	Drug Sensitivity Test		
	RIF		
	INH		
	EMB		
	STR		

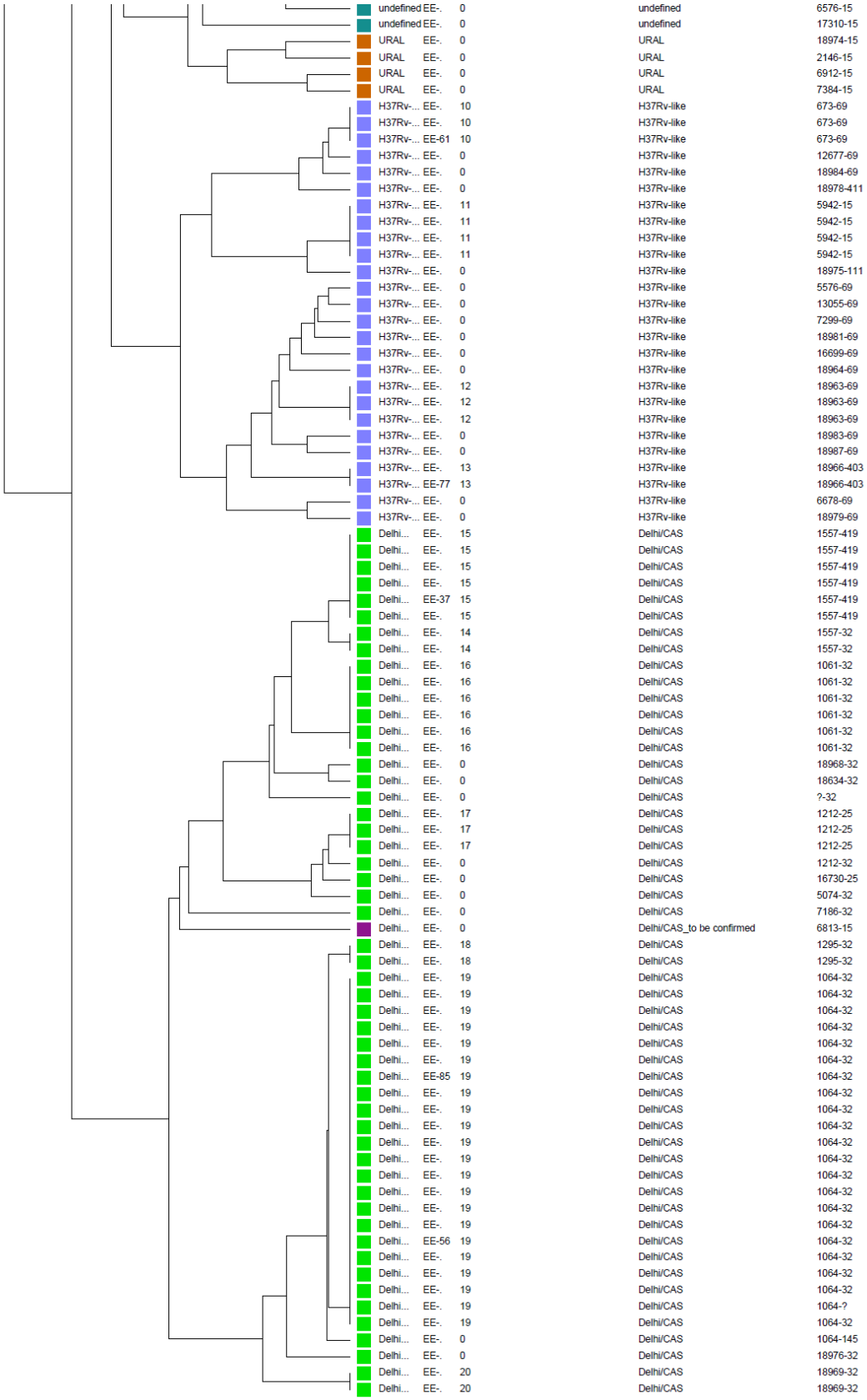
----------END----------

Thank you for your participation

Annex V: UPGMA tree for Genotyping and clustering of *M. tuberculosis*

UPGMA tree based on the copy numbers of MIRU-VNTR 24-loci and Spoligotyping, and it shows the pattern of drug resistance lineage and clustering of each genotype. The tree was calculated by using the MIRU-VNTR*plus* website




undefined EE-. 0 undefined 6576-15
undefined EE-. 0 undefined 17310-15
URAL EE-. 0 URAL 18974-15
URAL EE-. 0 URAL 2146-15
URAL EE-. 0 URAL 6912-15
URAL EE-. 0 URAL 7384-15
H37Rv-... EE-. 10 H37Rv-like 673-69
H37Rv-... EE-. 10 H37Rv-like 673-69
H37Rv-... EE-61 10 H37Rv-like 673-69
H37Rv-... EE-. 0 H37Rv-like 12677-69
H37Rv-... EE-. 0 H37Rv-like 18984-69
H37Rv-... EE-. 0 H37Rv-like 18978-411
H37Rv-... EE-. 11 H37Rv-like 5942-15
H37Rv-... EE-. 11 H37Rv-like 5942-15
H37Rv-... EE-. 11 H37Rv-like 5942-15
H37Rv-... EE-. 11 H37Rv-like 5942-15
H37Rv-... EE-. 0 H37Rv-like 18975-111
H37Rv-... EE-. 0 H37Rv-like 5576-69
H37Rv-... EE-. 0 H37Rv-like 13055-69
H37Rv-... EE-. 0 H37Rv-like 7299-69
H37Rv-... EE-. 0 H37Rv-like 18981-69
H37Rv-... EE-. 0 H37Rv-like 16699-69
H37Rv-... EE-. 0 H37Rv-like 18964-69
H37Rv-... EE-. 12 H37Rv-like 18963-69
H37Rv-... EE-. 12 H37Rv-like 18963-69
H37Rv-... EE-. 12 H37Rv-like 18963-69
H37Rv-... EE-. 0 H37Rv-like 18983-69
H37Rv-... EE-. 0 H37Rv-like 18987-69
H37Rv-... EE-. 13 H37Rv-like 18966-403
H37Rv-... EE-77 13 H37Rv-like 18966-403
H37Rv-... EE-. 0 H37Rv-like 6678-69
H37Rv-... EE-. 0 H37Rv-like 18979-69
Delhi... EE-. 15 Delhi/CAS 1557-419
Delhi... EE-. 15 Delhi/CAS 1557-419
Delhi... EE-. 15 Delhi/CAS 1557-419
Delhi... EE-. 15 Delhi/CAS 1557-419
Delhi... EE-37 15 Delhi/CAS 1557-419
Delhi... EE-. 15 Delhi/CAS 1557-419
Delhi... EE-. 14 Delhi/CAS 1557-32
Delhi... EE-. 14 Delhi/CAS 1557-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 16 Delhi/CAS 1061-32
Delhi... EE-. 0 Delhi/CAS 18968-32
Delhi... EE-. 0 Delhi/CAS 18634-32
Delhi... EE-. 0 Delhi/CAS ?-32
Delhi... EE-. 17 Delhi/CAS 1212-25
Delhi... EE-. 17 Delhi/CAS 1212-25
Delhi... EE-. 17 Delhi/CAS 1212-25
Delhi... EE-. 0 Delhi/CAS 1212-32
Delhi... EE-. 0 Delhi/CAS 16730-25
Delhi... EE-. 0 Delhi/CAS 5074-32
Delhi... EE-. 0 Delhi/CAS 7186-32
Delhi... EE-. 0 Delhi/CAS_to be confirmed 6813-15
Delhi... EE-. 18 Delhi/CAS 1295-32
Delhi... EE-. 18 Delhi/CAS 1295-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-85 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-56 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 19 Delhi/CAS 1064-?
Delhi... EE-. 19 Delhi/CAS 1064-32
Delhi... EE-. 0 Delhi/CAS 1064-145
Delhi... EE-. 0 Delhi/CAS 18976-32
Delhi... EE-. 20 Delhi/CAS 18969-32
Delhi... EE-. 20 Delhi/CAS 18969-32