Addis Ababa University

School of Graduate Studies

College of Natural Sciences

Department of Microbial, Cellular and Molecular Biology

Mycobacterium tuberculosis in central Ethiopia: Molecular Epidemiology, Drug Sensitivity Patterns, and Evaluation of the GenoType MTBDR*plus* Assay for the Detection of Rifampicin and Isoniazid Resistance

BY

Zufan Bedewi

A DISSERTATION SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITYIN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY (Biomedical Sciences)

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Abstract

Introduction: Identification of the types of strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) complex causing tuberculosis (TB), determination of their drug sensitivity patterns and rapid identification of MDR strains could contribute to TB control program.

Objective: the objective of this study was to identify the strains *M. tuberculosis* causing TB in central Ethiopia, determine their drug sensitivity patterns, and evaluate of the GenoType MTBDRplus assay for detecting of Rifampicin and Isoniazid resistance.

Methodology: A total of 338 *M. tuberculosis* complex were isolated from pulmonary TB cases in central Ethiopia and 297 culture positive isolates were included in this study. The isolates were analyzed using region of difference (RD) 9-based polymerase chain reaction (PCR) and spoligotyping. Additionally, mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) analysis was done on 29 isolates. The drug sensitivity pattern of the isolates was performed using conventional and molecular assay. Evaluation of MTBDR*plus* assay was done using conventional method as a gold standard.

Results: The Mycobaterium isolates were majorly *M.tuberculosis* (98.6%) while only 1.4% was *M. africanum*. Ninety different patterns were identified, 45 were registered in SpolDB4 while 45 were not found in the database. Of these 90 spoligotypes, 32 were clustered consisting of 223 (79.3%) isolates while 58 had only a single isolate each. The dominant spoligotypes were SIT 53, SIT 149, and SIT 54 consisting of 43, 37 and 34 isolates, respectively. Majority (86.8%) of the isolates belonged to the Euro-American lineage followed by East-African-Indian (6.4%) and Indo-oceanic (5%) lineages. The result of MIRU-VENTR showed that isolates with similar spolygotype pattern showed difference in their copies of 24 MIRU loci. Of the 268 *M. tuberculosis* isolated from new cases, 59 (22.2%) were resistant to at least one of the first line drugs. The prevalence of multi-drug resistant (MDR) *M. tuberculosis* in new TB cases was 1.5% (4/268). The sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of Rifampicin resistant *M. tuberculosis* were 80.0% and 99.6%, respectively while they were 82.7% and 99.6%, respectively for the detection of Isoniazid (INH) resistant *M. tuberculosis.* Its sensitivity and specificity in detecting MDR *M. tuberculosis* were 75.0% and 100%, respectively, and its concordance with the conventional drug sensitivity test was 81.4%.

Conclusion: The main cause of TB in human in central Ethiopia was *M. tuberculosis*. The majority of the *M. tuberculosis* isolates were found in clustered spoligotypes, which could suggest the possibility of on-going transmission in the study areas. A significant proportion of *M. tuberculosis* was resistant to at least one first line anti-TB drug warranting for improving the control program. GenoType MTBDR*plus* assay had an acceptable sensitivities and specificities for the detection of Rifampicin and Isoniazid resistant *M. tuberculosis*. Moreover, it had good concordance with the conventional drug sensitivity test suggesting its potential application for drug sensitivity test.

Keywords: Central Ethiopia, Drug sensitivity, GenoType MTBDR*plus* assay, Proportion method, *M. tuberculosis*, Strain diversity

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Abbreviations

AM	Amikacin
ATP	Adenosin tri phosphate
bp	Base pair
CAP	Capromycin
CBN	Conformal Bayesian network
CFX	Ciprofloxacin
CI	Confidence intervals
CRISPRs	Clustered Regularly Interspaced Short Palindromic Repeats region
DNA	Deoxo-nuclic acid
DR	Direct repeats
DST	.Drug susceptibility testing
EA	Euro-American
EAI	East-African -indian
EMB	Ethambutol
ETO	Ethionamide
HIV/AIDS	Human immunodeficiency virus
INH	Isoniazid
IO	Indo-occenic
IS	Insertion sequence
INH	Isoniazid
I(FASI)	Inhibition of fatty acid synthase
KAN	Kanamycin
KBBN	Knowledge based Bayesian network
LiPA	Line probe assay

LSPs	Large Sequence Polymorphism
LJ	Löwenstein –Jensen medium.
MA	M.-africanum
MIC	The minimum inhibitory concentration
MGIT	Mycobacteria growth indicator tube
MUT	Mutation
mRNA	Messenger RNA
Mtb	*Mycobacterium tuberculosis*
MDR-TB	Multi-drug resistant tuberculosis
MTBC	*Mycobacterium tuberculosis* complex
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number tandem repeat
NTM	Non-tuberculous mycobacteria
OP	Optical density
OR	Odds ratios
PCR	Polymerase chain reaction
PZA	Pyrazinamide
QRDR	Quinolone resistance-determining region
RFLP	Restriction fragment length polymorphism
RD	Regions of Difference (RDs)
RIF	Rifampicin
RRDR	Rifampicin resistance-determining region
rRNA	Ribosdmal RNA
RNA	Ribonucleic acid
RNAP	DNA-dependent RNA polymerase

SIT	Shared international type
SNPs	Single nucleotide polymorphisms
STM	Streptomycin
TbD1	Tuberculosis deletion 1
TB	Tuberculosis
UPGMA	Un weighted-pair group method with arithmetic averages
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug resistant TB

CHAPTER ONE

1. Introduction

Tuberculosis (TB) is one of infectious disease, affecting millions of people worldwide. TB is caused by a group of phylogenetic ally closely related bacteria, collectively known as the *Mycobacterium tuberculosis* complex (MTBC). Although there is an effective treatment for decades, TB remains a major global health problem and affects mostly economically productive age groups. According to World Health Organization report two-thirds of cases are estimated to occur among young people (WHO, 2013).

Worldwide, two billion people are latently infected with *Mycobacterium tuberculosis* (*Mtb)*, providing a large reservoir for active TB that will last for decades (Barry *et al.*, 2009). In 2014, there were an estimated 9.6 million new TB cases of this, an estimated 5.4 million were men, 3.2 million were women and 1.0 million were children (WHO, 2015). There were also 1.5 million TB deaths, of which approximately 890,000 were men, 480,000 were women and 140,000 were children (WHO, 2015). The number of cases is estimated to be 12 million worldwide (WHO, 2015). The South-East Asia and Western Pacific Regions collectively accounted for 58% of the world's TB cases in 2014 (WHO, 2015). The African Region had 28% of the world's cases (WHO, 2015). India, Indonesia and China had the largest numbers of cases, 23%, 10% and 10% of respectively (WHO, 2015).

The spread of human immunodeficiency virus (HIV) and drug-resistant TB have aided in posing serious healthcare. Drug resistance TB threatens the National TB Control Programs in several countries. One in three patients infected with HIV has TB, and drug resistance is emerging at an alarming rate in some geographical areas.

Globally in 2014, there were an estimated 1.2 million HIV co-infected new TB cases (12% of all TB cases) (WHO, 2015). Almost three-quarters of these cases were in the African Region (WHO, 2015). Of 1.5 million TB deaths, 390,000 TB deaths occur among HIV-positive people (WHO, 2015).

Worldwide, an estimated 480,000 people having developed MDR-TB in 2014, the proportion of new cases with MDR-TB was 3.3% and 20% of previously treated TB case (WHO, 2015). On average, an estimated 9.7% of patients with MDR-TB had extensively drug resistant TB (XDR-TB) (WHO, 2015). More than half of the MDR-TB cases found in three countries alone: India, China and the Russian Federation (WHO, 2014; WHO, 2015).

There are about 22 countries with a high TB burden which are collectively accounted for 82% of the global TB burden (WHO, 2014). The countries with the highest incident cases in 2013 were India (2.0 million–2.3 million), China (0.9 million–1.1 million), Nigeria (340 000−880 000), Pakistan (370 000−650 000), Indonesia (410 000−520 000) and South Africa (410 000−520 000). Of the estimated 1.2 million cases co-infected with HIV, 78% were living in Africa. In parts of southern Africa, more than 50% of TB cases were co-infected with HIV (WHO, 2014).

Ethiopia has high incidence rate of TB infection and the disease is one of major public health problems in the country. The country is one among the world's 22 countries, with high TB burden (WHO, 2014). The annual incidence of new TB cases was estimated to be 207/100,000 and the prevalence of TB was 200/100,000 (WHO, 2015). The country is one of the 27 countries with a high MDR-TB burden; ranked 15th among them (WHO, 2014). In 2014, TB mortality was estimated to be 33 per 100,000 of the population (WHO, 2015). The same report showed that 1.6% of new TB patients and 12% of previously treated patients had MDR-TB.

TB is caused by a group of phylogenetically closely related bacteria, collectively known as the *Mycobacterium tuberculosis* complex (MTBC). In humans it is mostly caused by the members of the MTBC known as *Mycobacterium tuberculosis* and *Mycobacterium africanum.*

According to whole genome sequence analyses, MTBC consist seven human-adapted lineages (Lineages 1–7). The TbD1-deleted modern clad consist Lineages 2, 3 and 4 and the ancestor one without the deletion of TbD1are lineage 1, 6 and7. Among these lineage 2(East-Asian lineage includes the Beijing family) and lineage 4(Euro-American) diversified more recently than the remaining MTBC strains. Lineage 2 predominates in East Asia, but is also present in Central Asia, Russia and South-Africa. Lineage 4 mostly occurs in Asia, Europe, Africa and America (Bos *et al*.2014). The "ancestral" strains Lineages 1 and modern strain lineage 3 show a more restricted geographical distribution limited to East Africa, Central-, South and South-East Asia. The most geographically restricted lineages are Lineages 5–7. Lineages 5 and 6 are also known as *M. africanum* West Africa 1 and West Africa 2, respectively, and almost totally occur in West Africa or in recent immigrants from those regions (de Jong *et al.,* 2010). Similarly, the recently discovered Lineage 7 is limited to Ethiopia (Firdessa *et al.,* 2013).

Strain diversity in MTBC has important phenotypic consequences. This genotyping diversity is produced by mutations which occur across the genomes, and produced by Gene duplications, Single nucleotide polymorphisms, gene deletions and repetitive and mobile genetic elements. The genotyping diversity of MTBC can have an impact on bacterial virulence phenotypes (Gehre *et al* 2013). Different MTBC strains and lineages differ in their growth, Immunogenicity, disease severity, disease

presentation, disease transmission and also differ in their gene expression and metabolic profiles.

Strains of MTBC differ in their content of SNPs, like small insertion or deletions, large genomic deletions, large duplications and insertion sequences. There are different molecular tools which are important to differentiate these changes and to form phylogenetic classification. These are Restriction Fragment Length Polymorphism typing, spoligotyping, variable number of tandem repeat analysis or Mycobacterial Interspersed Repetitive Units, long sequence polymorphism (genome deletion analysis) and Whole Genome sequencing. Of these different molecular tools for better differentiation of related strains Whole Genome sequencing is preferred which provides the best discriminatory power to differentiate between strains. Moreover, Whole Genome sequencing allows computing phylogenetic distances and quantifying genomic diversity within and between groups of strains.

1.1 Significance of the study

Effective TB control programs require the initiation of appropriate therapy as well as an understanding of the epidemiology of the disease (Huard *et al*., 2003). Moreover, monitoring the emergence of drug resistant strains is very important and information based on the drug susceptibility pattern of the TB bacteria is used as an indicator for TB treatment quality. Understanding the drug susceptibility pattern of the isolates and its relationship to the bacteria strain is important to detect if drug resistance is influenced by the genetic and evolutionary background of MTBC strains.

Such type of study is important to identify the anti TB drug resistant strains, to understand their spread, to control their distribution and to detect new outbreaks. Such studies also are helpful to provide information which will support modification of health policy and to develop recommendations which is important impute for the public health authorities to control drug resistance TB. Information generated during such type of study will be publicized which is important to protect the inappropriate use of anti-TB drugs and it is useful to detect treatment quality.

The central Ethiopia expected to have high TB case like the other part of the country because of higher population flow and over-crowding. However, there is a scarcity of data on detection of drug resistance, identification of drug resistant strains and identifying the most dominant strains. To our knowledge there is no data on strain diversity of *M. tuberculosis* circulating in south and southwestern part of central Ethiopia. Thus, it is important to identify the types of strains of MTBC causing TB and MDR-TB in this specific geographic region of Ethiopia which could contribute to the strengthening the TB Control Program of the country.

1.2. Objectives

1.2.1. General Objective: The aim of this study is to evaluate molecular epidemiology and drug susceptibility patterns of MTB isolated from central Ethiopia.

1.2.2. Specific Objectives:

- To identify the strains of *M. tuberculosis* causing TB in three towns of central Ethiopia and their surroundings
- To evaluate the drug sensitivity patterns of the *M. tuberculosis* isolated from three towns of central Ethiopia and their surroundings
- To investigate the association between drug resistance and the strains of *M. tuberculosis*
- To evaluate the performances of the Genotype MTBDR*plus* assay for the detection resistance to Rifampicin and Isoniazid using the conventional LJ-based proportion method as a gold standard

CHAPTER TWO

2. Literature Review

2.1 *Mycobacterium tuberculosis* complex (MTBC)

The *Mycobacterium tuberculosis* complex consists of a group of acid-fast Mycobacteria which cause tuberculosis diseases (Cole, 2002). This group comprises the so-called *M. tuberculosis, M. bovis, M. microti, M. africanum, M. pinnipedii,* and *M. caprae* species. Members of the *M. tuberculosis* complex are highly related mycobacteria exhibiting remarkable nucleotide sequence level homogeneity despite varying in pathogenicity, geographic range, certain physiological features (such as colony morphology as well as profiles of resistance and susceptibility to inhibitors), epidemiology and host preference (Frothingham *et al.*, 1998).

The species of *M. tuberculosis* and *M. africanum* are human pathogens, but sometimes they cause disease in other primates and animals (Cole, 2002). Several animal-adapted members of MTBC exist, which affect a range of wild and domestic animal species (Smith *et al.*, 2005). These include *M. bovis* (a pathogen of cattle), *Mycobacterium caprae* (sheep and goats), *Mycobacterium microti* (voles) and *Mycobacterium pinnipedii* (seals and sea lions). *Mycobacterium bovis* used to be a significant cause of human TB, primarily in people who consumed raw milk (Grange, 2001). *M. canettii*, the ancestor MTBC cause diseases in humans and it is a part of the so-called 'smooth TB bacilli' first described in the 1960s and so far only about 60 isolates have been reported (Fabre *et al.*,2010). The large majority of these were recovered from TB patients in Djibouti or from individuals who spent some time at the Horn of Africa. *Mycobacterium canettii* produces smooth and shiny colonies and harbour much more genetic diversity compared with classical MTBC. Most

importantly, *M. canettii* shows clear evidence of ongoing horizontal gene exchange, which does not occur in other MTBC (Hirsh *et al*., 2004). Because of these unique characteristics of *M. canettii* studies assumed that this organism is an opportunist, and that an environmental reservoir exists somewhere in the Horn of Africa (Koeck *et al*., 2010).

Several studies showing that, many of the genes among the different members of MTBC bacilli are identical. At whole genome level, the species of the MTBC share greater than 99% of DNA identity (Brosch *et al*., 2000). Furthermore, DNA sequence analysis of the MTBC isolates have revealed that allelic polymorphism is extremely restricted, occurring in 1 in 10,000 base pairs, significantly less compared to other pathogenic bacteria and the absence of horizontal gene transfer, unlike the majority of bacterial pathogens (Arnold, 2007).

These polymorphic genome sites are used, for classification of MTBC strains, for epidemiological investigations, for phylogenetic study and for the study of pathogenesis and drug sensitivity test. The polymorphic genome sites of MTBC are divided into three main groups, i.e., single nucleotide substitutions, long sequence polymorphisms, and polymorphisms in repetitive sequences. The latter are subdivided into scattered repeats (direct repeats, e.g., the DR region, and insertion repeats, e.g., the IS element) and tandem repeats (direct continuous repeats).

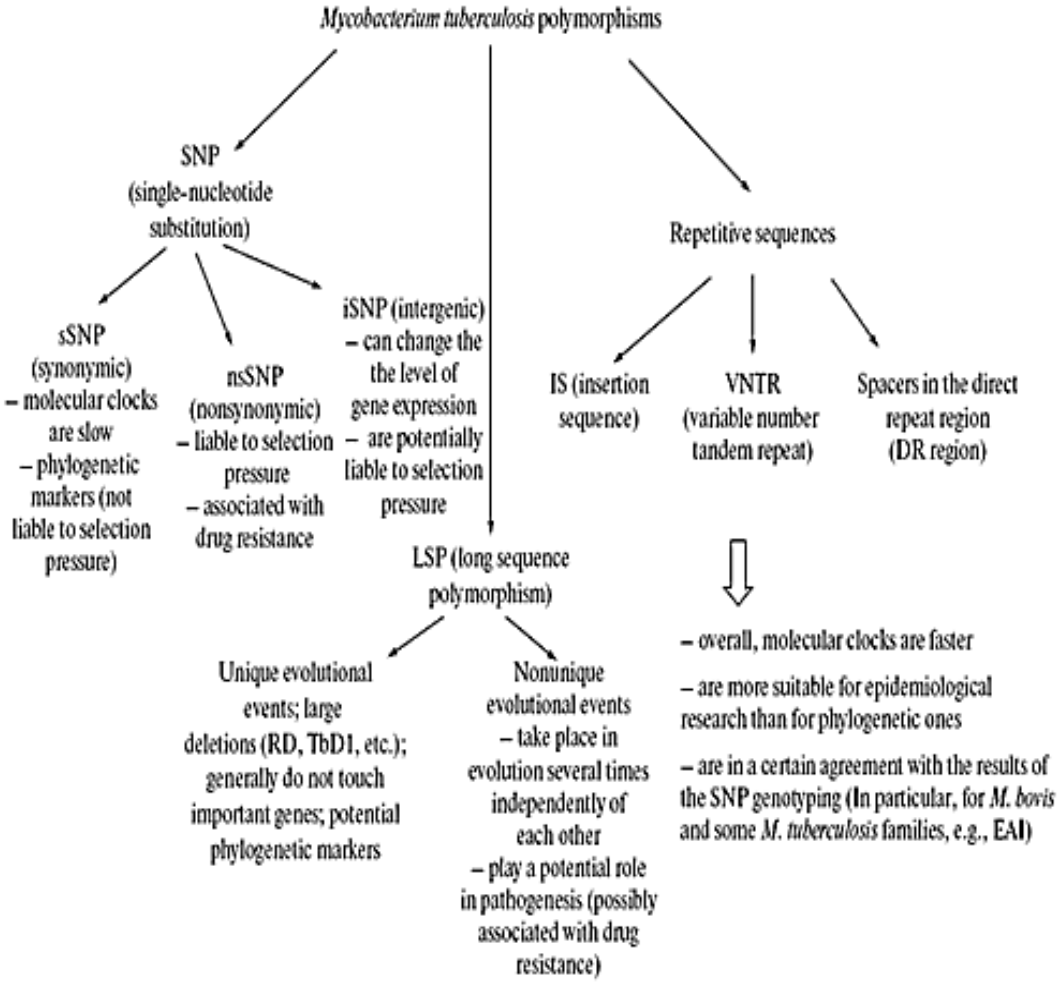

Figure 2.1 classifications of polymorphisms in the *M. tuberculosis* (Supply *et al*., 2000).

2.2 Molecular epidemiology of *Mycobacterium tuberculosis* complex.

Molecular typing methods of *M tb* has greatly improved the knowledge and control of TB by allowing, the detection of unsuspected transmission, the identification of false positive cultures, the distinction between re-infection and relapse, the differentiation of MTBC to species or strain level, the study of drug susceptibility pattern among *M.tb* isolates and also be used to evaluate an outbreak of TB. If epidemiologic data suggest the occurrence of an outbreak, genotyping of the isolates, in combination with an epidemiologic investigation, can help determine whether an outbreak has occurred or whether there is a coincidental occurrence of a large number of cases. This strategy can delineate the extent of the outbreak and guide public health measures to reduce disease transmission (Barnes and Cave, 2003).

In addition DNA fingerprinting generated huge amount of genetic data which have been used to study the phylogeny and evolution of the MTBC members. Analyses by various genetic markers indicate that *M. tuberculosis* evolves and disseminates by clonal expansion (Warren *et al.*, 2001; Supply *et al.*, 2003; Baker *et al.*, 2004) which results in great geographic variations in the distribution of *M. tuberculosis* evolutionary lineages (Filliol *et al.*, 2002; Baker *et al.*, 2004).

A good genotyping method should assess a marker that remains stable during the study period, and does not vary to a degree that confuses the epidemiological picture. This marker should be testable in every isolate, i.e., it should provide universal type ability of all isolates. It should also usefully discriminate among isolates, and this discrimination should be concordant with the epidemiological picture. Finally, the

results of a good typing method should be reproducible, independently of the operator, place and time (Kanduma *et al*., 2003).

Several molecular techniques can be used as a tool for studying molecular epidemiology of *Mycobacterium tuberculosis*. Among them, IS6110 restriction fragment length polymorphism (RFLP) typing (van Embeden 1999), spoligotyping (Kamerbeek *et al*., 1997), mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing (Mazars *et al*., 2001), region of difference and single nucleotide polymorphism are the most widely used.

2.2.1. IS6110 restriction fragment length polymorphism (RFLP) for studying molecular epidemiology of tuberculosis

This typing method is based on the identification of the presence of so called insertion sequences (IS), i.e., mobile genetic elements with lengths of up to 2500 bp, which are widely distributed in most bacterial genomes. The mobile insertion sequence IS*6110* was first identified from the chromosome of *M. tuberculosis* in1990 (Thierry *et al*., 1990a) and was found to be specific to the members of the MTBC (Cave *et al*., 1991). The number of copies of IS elements (IS6110) varies from 1 to 26 in different *M. tuberculosis* strains. It works by an analysis of the **r**estriction fragment length polymorphism (RFLP).

IS*6110*-based genotyping requires sub culturing the isolates for several weeks to obtain sufficient DNA (van Soolingen, 2001) and the DNA from the bacterial culture is first cuts using common *Pvu*II restriction endonuclease then undergoes the hybridization of restriction fragments with an IS6110 probe. This probe then

specifically marks those fragments, which contain the repetitive element IS*6110* (Seidler *et al.*, 2004). The number of resulting bands corresponds to the number of IS*6110* copies. These patterns may be very different between epidemiologically unlinked isolates, but the banding patterns of serial isolates from an individual are relatively stable over (Seidler *et al*., 2004).

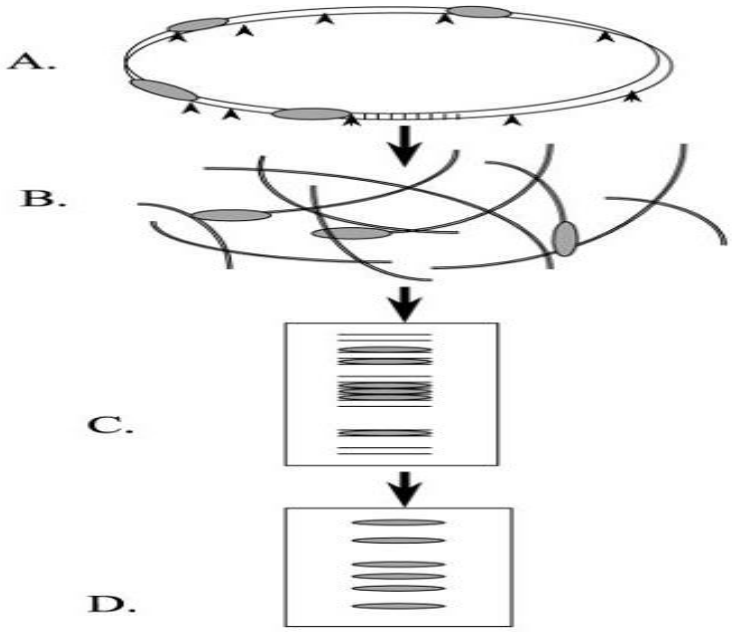

Figure 2.2**.** Genetic fingerprinting of *M. tuberculosis* isolates by RFLP

(A) Restriction enzymes cleave chromosomal DNA at restriction sites (arrowheads).

(B) Some DNA fragments contain IS*6110* (repetitive sequences of base pairs, represented as shaded ellipses).

(C) The fragments are separated according to size by gel electrophoresis.

(D) Fragments containing IS*6110* hybridize to the specific radioactive probe, which produces a characteristic banding pattern (fingerprint) for each isolate (Kulaga *et al*., 1999).

The patterns of IS6110 RFLP genotyping were fairly stable with respect to time but simultaneously quite polymorphic, which allows them to be used to study TB transmission dynamics at the local and population levels. The IS6110 RFLP method ensures a very high resolution when analyzing the transmission of *Mycobacterium tuberculosis* strains and was an international gold standard in mycobacterial genotyping until the middle of the 2000s. A serious disadvantage of this method is its limited resolving capacity in analyzing clinical strains with six or less IS6110 copies .In addition, this method is exigent to laboratory equipment, as well as to the quantity of DNA and quality of its purification, more laborious, and takes much time (Das *et al.*, 2004).

2.2.2 Typing by spacer oligo nucleotides (Spoligotyping) for studying molecular epidemiology of tuberculosis

Spoligotyping is typing method based on the presence or absence of 43 unique regions called spacers that separate direct repeats (DRs) in the Clustered Regularly Interspaced Short Palindromic Repeats region (CRISPRs) of the MTBC genome.

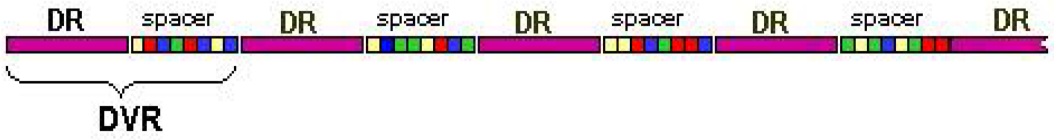

Figure 2.3 .DR loci (fragment). 43 spacers are used in spoligotyping assay Kamerbeek *et al.*, 1997.

By spoligotyping one can detect the presence or absence of spacers of known sequence. This is accomplished by the PCR amplification of the DR region that is

derived from the DR sequence. Then be hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequence of *M. tuberculosis* H37Rv and *Mycobacterium bovis* by reverse line blotting (van Soolingen, 2001). The digital format of spoligotyping data has enabled a worldwide computer base of spoligotypes to be created that is constantly updated. The recently created fourth version (SpolDB4) contains several thousand spoligotypes isolated from patients around the world. Spoligotyping reveals strains with the low number of IS6110 copies (Bauer *et al.*, 1999).The results of the spoligotyping method can be reproduced well, as well as be easily interpreted and computerized and can be read as a digital code even in a word processor (Arnold *et al.*, 2007). However, the resolving capacity of spoligotyping is as much lower than that of IS6110 RFLP typing.

Spoligotyping can be used for prescreening and it is well suited to primarily analyze the population structure of *M. tuberculosis* and to determine the main genetic groups (families). Spoligotyping is useful both for tracking epidemics (Kamerbeek *et al.*, 1997), for the description of highly prevalent families such as the Beijing family and for detecting new outbreaks and better defining high-risk populations in order to focus prevention strategies on the subpopulations that need them most (van Soolingen, 2001; Ahmed and Hasnain, 2004).

Table 2.1: Diagnostic spoligo spacer missing for *M. tuberculosis* family members (12), *M. africanum* and host adapted *M. bovis* strains (35) (Smith *et al*., 2005).

*M. tb*family members	Spacer lacking
M. tb(Beijing)	1-34
M. tb(Haarlem)	31, 33-36
M. tb(Latin America)	21-24, 33-36
M. tb(East African India)	29-32, 34
M. tb(Central Asia)	4-7, 23-34
M. tb(Cameroon)	23-25, 33-36
M. africanum(Type I)	9, 39
M. bovis(antelope)	9, 16, 39
M. bovis(seal/vole)	3, 9, 16, 39-43
M. bovis(caprine)	3, 9, 16, 39-43
M. bovis(cattle)	3, 9, 16, 39-43
*M. bovis*BCG	3, 9, 16, 39-43

2.2.3 Mycobacterial Interspersed Repeat Unit- Variable Number Tandem Repeats (MIRU-VTR) for studying molecular epidemiology of tuberculosis

Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats (MIRU-VNTR) are loci which show hyper-variability in their repeat numbers in *M. tuberculosis* complex. (Mazars *et al*., 2001). In 2000, Supply *et al* discovered and analyzed 41 loci of this type in the *M. tuberculosis* genome (Supply *et al*., 2000); 24

of these loci were identified as hyper variable repetitive units (Frothingham *et al.*, 1998).

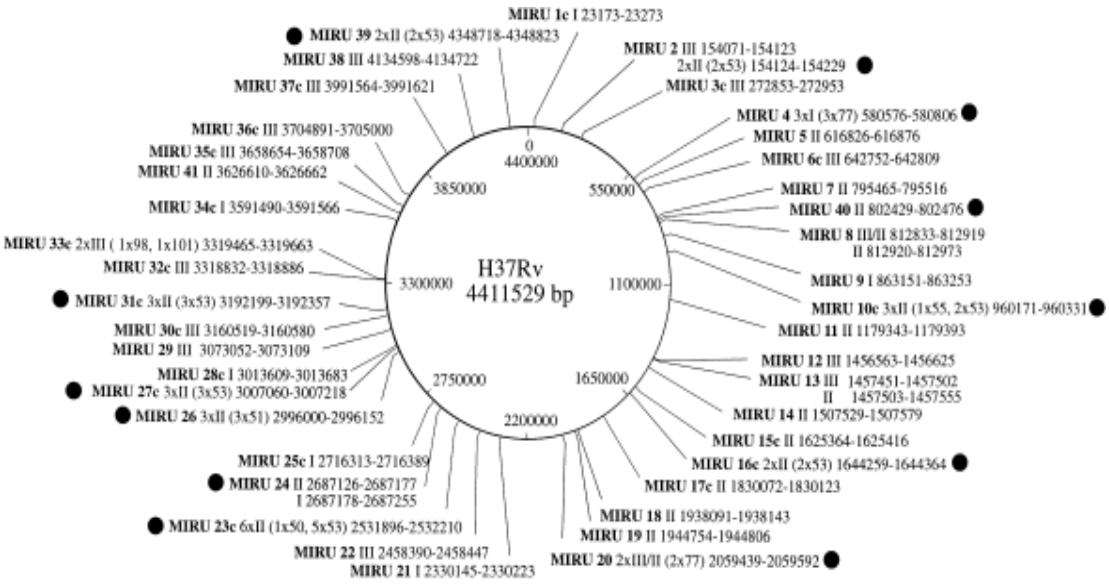

Figure 2.4.Position of the 41 MIRU loci on the *M. tuberculosis* H37Rv chromosome (Supply *et al*., 2000).

MIRUs are short DNA elements often found as tandem repeats and dispersed in intergenic regions of the genome of the *M. tuberculosis* complex (van der Spuy *et al*., 2003). The different strains vary in the number of repeats at different loci. Each typed strain is assigned a 24 digit number, forming the basis of a coding system that facilitates inter laboratory comparisons (Allix *et al.*, 2004).

The MIRU-VNTR genotyping technique is a PCR-based method mostly uses 24 different intergenic regions for genotyping. Calculation of the number of repeats is performed, on the basis of the amplicon size. MIRU results are reported as 24

character designations, each of them corresponds to the number of repeats at one of the 24 loci (Mazars *et al.*, 2001 and Supply *et al.*, 2006). It is reproducible, sensitive and specific for MTBC isolates. It is highly suitable for global epidemiological surveillance of tuberculosis (Kanduma *et al*., 2003).

MIRU-VNTR genotyping technique is fast, appropriate for strains identification; it is more efficient and less labor intensive, as well as much less exigent to the quality of DNA. This method can be automated by analyzing the lengths of PCR fragments using automatic sequencers, which significantly increases the efficiency of genotyping (Mazars *et al.,* 2001). The data on the number of repeats of each locus are easily compared inside and inter laboratories. International databases on the MIRU-VNTR profiles of mycobacteria have been created and are constantly updated.

The apparent association of the number of allele variants with the genetic groups of mycobacteria enables the results of the MIRU-VNTR typing to be used as ersatz marker of phylogenetic groups (supply *et al.,* 2001). Preliminary studies suggest that although MIRU-VNTR gives a higher degree of discrimination than spoligotyping, best results are obtained by combining the two techniques (Durr *et al*., 2000). Spoligotyping and Mycobacterial Interspersed Repeat Units (MIRUs) typing together have been recently defined as the new gold standard for molecular epidemiological investigation of TB (Jagielski *et al*., 2014).With this multilocus method, the isolates can be typed for practical epidemiological purposes, and simultaneously, the evolutionary relationships between strains can be defined to identify the genotype family to which the strain belongs (Supply *et al*., 2001). The data of MIRU-types can be compared using the SITVITWEB database that includes thousands of MIRU-VNTR *plus* (Weniger *et al.,* 2010).

2.2.4 Genomic deletions for studying molecular epidemiology of tuberculosis

This typing method is using the genomic deletions of the bacteria to differentiate MTBC members. The genomic deletions often referred to as Regions of Difference (RDs) or Large Sequence Polymorphism (LSPs), which have been used as markers to classify groups of MTBC strains into main phylogenetic lineages (Hirsh *et al.*,2004; Mostowy *et al*., 2004; Tsolaki*et al*.,2004; Gagneux *et al.*, 2006), and sub-lineages (Tsolaki *et al*., 2005 and Alland *et al*., 2007) . Because on-going horizontal gene exchange is rare in MTBC, LSPs are essentially irreversible, making them ideal phylogenetic markers for strain classification. Large sequence polymorphisms (LSPs) were most often resulting from genomic deletions and regroupings. The loss of a genome site in an ancestral gene can serve as a genetic marker for the progenitor of the genotype of the strain under investigation. By using deletion sites as genetic markers, it is possible to carry out the analysis by the simple PCR or by the automatic Gene Chip technology. The analysis of chromosome deletions is an important tool in studying the global evolution and phylogeny of the disease.

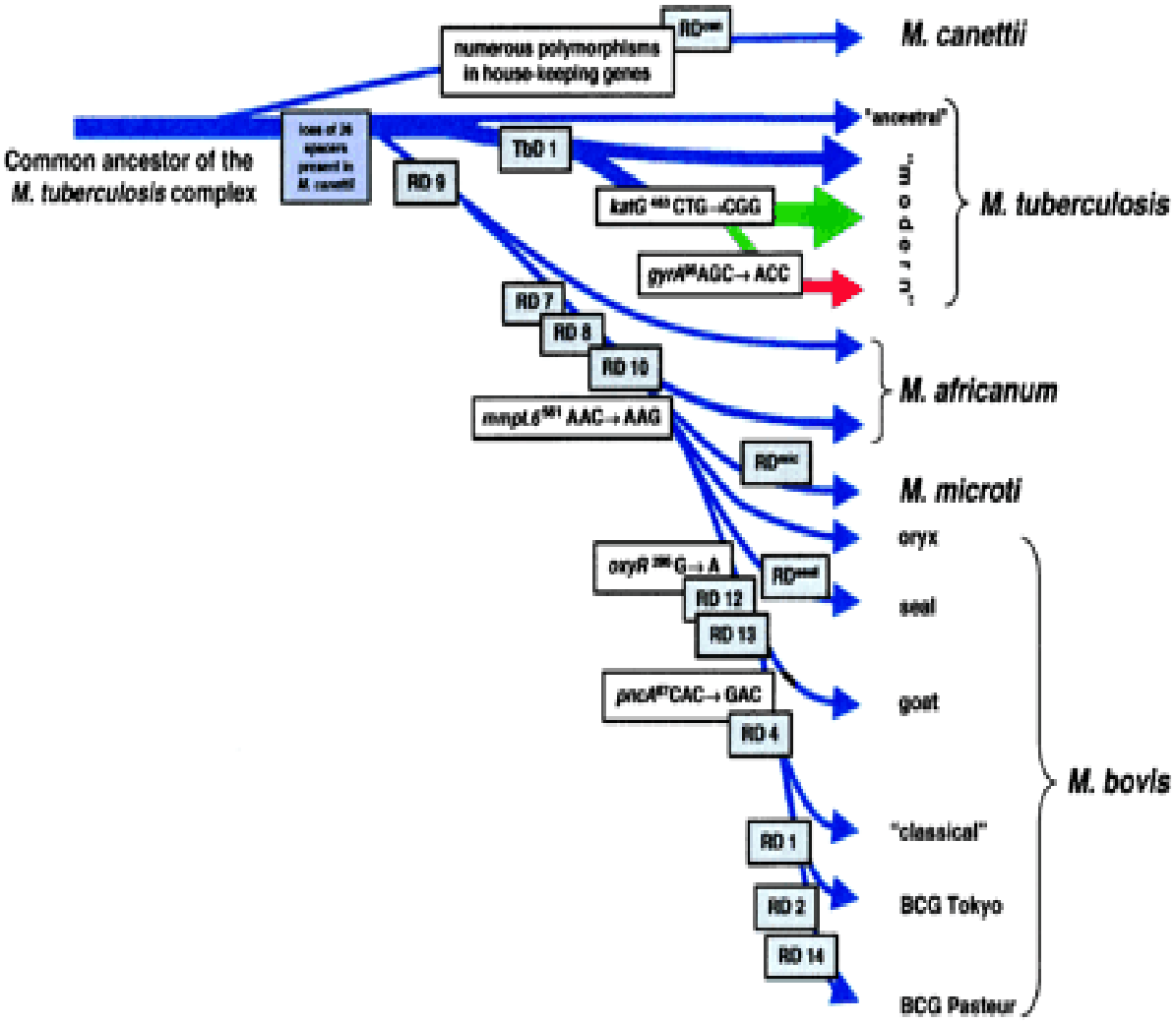

Figure 2.5 Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (Brosch *et al*., 2002)

2.2.5 Single nucleotide polymorphisms (SNPs) for studying molecular epidemiology of tuberculosis

An analysis of single nucleotide substitutions can be used in phylogenetic and population genetic, as well as molecular epidemiological, studies on drug resistance and host pathogen interactions (Baker *et al*., 2004). The detection of substitutions can be automated using either real time PCR or sequencing. By this method the detailed phylogeny of the *M. tuberculosis* complex in which the relations of other phylogenetic ersatz markers were also determined. SNPs can be classified as synonymous, non-synonymous, nonsense, or intergenic. Non synonymous SNPs are very important types which cause the substitution of an amino acid; therefore, they are vulnerable to the effects of natural selection and major contributors to functional mutations. They are considered to be keys in the mechanism of the origin of drug resistance in *M. tuberculosis.* Resistance to anti-TB drugs is associated to single nucleotide mutations that occur in the drug targeted genes. These mutations reduced the susceptibility to a particular drug by either modifying the drug-target, increasing the expression of the gene product targeted by the drug, or by reducing the drug activation in the case of pro drugs (Trauner *et al*., 2014). In addition to drug resistance non synonymic SNPs causes in changing of bacterial virulence. Study showed that non-synonymous SNPs or point mutation in one of the MTBC virulence gene i.e. PhoPR component lead to important changes in bacterial phenotype (Ryndak *et al*., 2008). For insistence, one amino acid change at position 219 of PhoP in the laboratory strain H37Ra alters the binding capacity of PhoP to its own promoter (Chesne-Seck *et al*., 2008.), as a result, H37Ra is highly attenuated compared to virulent H37Rv.

2.3 Phylogeny of human-adapted *Mycobacterium tuberculosis complex*

According to whole genome sequence analyses, MTBC consist seven human-adapted lineages (Lineages 1–7) each associated with specific global geographical locations (Comas *et al*., 2013).These lineages grouped as modern lineage, with a deletion gene in the genomic region known as tuberculosis deletion 1(TbD1) and others known as ancestral strains, without this deletion (Brosch *et al*., 2002). The TbD1-deleted modern clad consist Lineages 2, 3 and 4. Among these lineage 2 (East-Asian lineage includes the Beijing family) and lineage 4 (Euro-American) diversified more recently than the remaining MTBC strains. Lineage 2 predominates in East Asia, but is also present in Central Asia, Russia and South-Africa. Lineage 4 mostly occurs in Asia, Europe, Africa and America.

The "ancestral" strains Lineages 1 and modern strain lineage 3 show a more restricted geographical distribution limited to East Africa, Central-South- and South-East Asia (Fig.2.6). The most geographically restricted lineages are Lineages 5, 6 and 7, which are all associated with specific regions of Africa. Lineages 5 and 6 are also known as *M. africanum* West Africa 1 and West Africa 2, respectively, and almost exclusively occur in West Africa or in recent immigrants from those regions (de Jong *et al.,* 2010). Lineage 6 occurs primarily in the Western part of West Africa, whereas Lineage 5 dominates further to the East in regions bordering the Gulf of Guinea (Gehre *et al.,* 2013a). Similarly, the recently discovered Lineage 7 is confined to Ethiopia and recent immigrants from the country (Firdessa *et al.,* 2013). The reasons, why these three lineages are limited to specific regions of Africa are unknown.

In addition Africa is the only region of the world that contains all seven main human-adapted MTBC lineages and harbours the largest diversity of these lineages (Gagneux

and small, 2007). Modern humans; i.e. *Homo sapiens* from Africa are known to be phylo-genetically 'ancestral' and harbour most of the known human genetic diversity (Tishkoff *et al*.,2009). Human-adapted MTBC exhibits a phylo geographic population structure with different lineages associated with different human populations (Gagneux *et al* .,2006). Based on the different studies, the origin and global spread of human TB might be started from Africa (Hershberg *et al*., 2008). This Assumption was supported by multi-locus sequence data from 108 global MTBC strains and hypothesizes that human MTBC originated in Africa and accompanied out-of-Africa with the migrations of modern human population (Hershberg *et al*., 2008). Most MTBC lineages left Africa and spread into Europe and Asia but two or three phylogenetically 'ancient' MTBC lineages stayed in Africa (Comas *et al*., 2013). The MTBC populations in Europe and Asia expanded, and in parallel spread globally through the waves of human exploration, trade and occupation.

Recently, a new group of MTBC has been identified in 1000-year-old human remains from Peru (Bos *et al.,* 2014). These ancient MTBC strains were distinct from any known human-adapted MTBC, but most closely related to contemporary *M. pinnipedii* which is adapted to seals and sea lions. These findings suggest that marine mammals could have played a role in spreading TB from Africa across the Atlantic Ocean to the New World and transmitting to pre-Columbian human populations (Bos *et al.,* 2014).

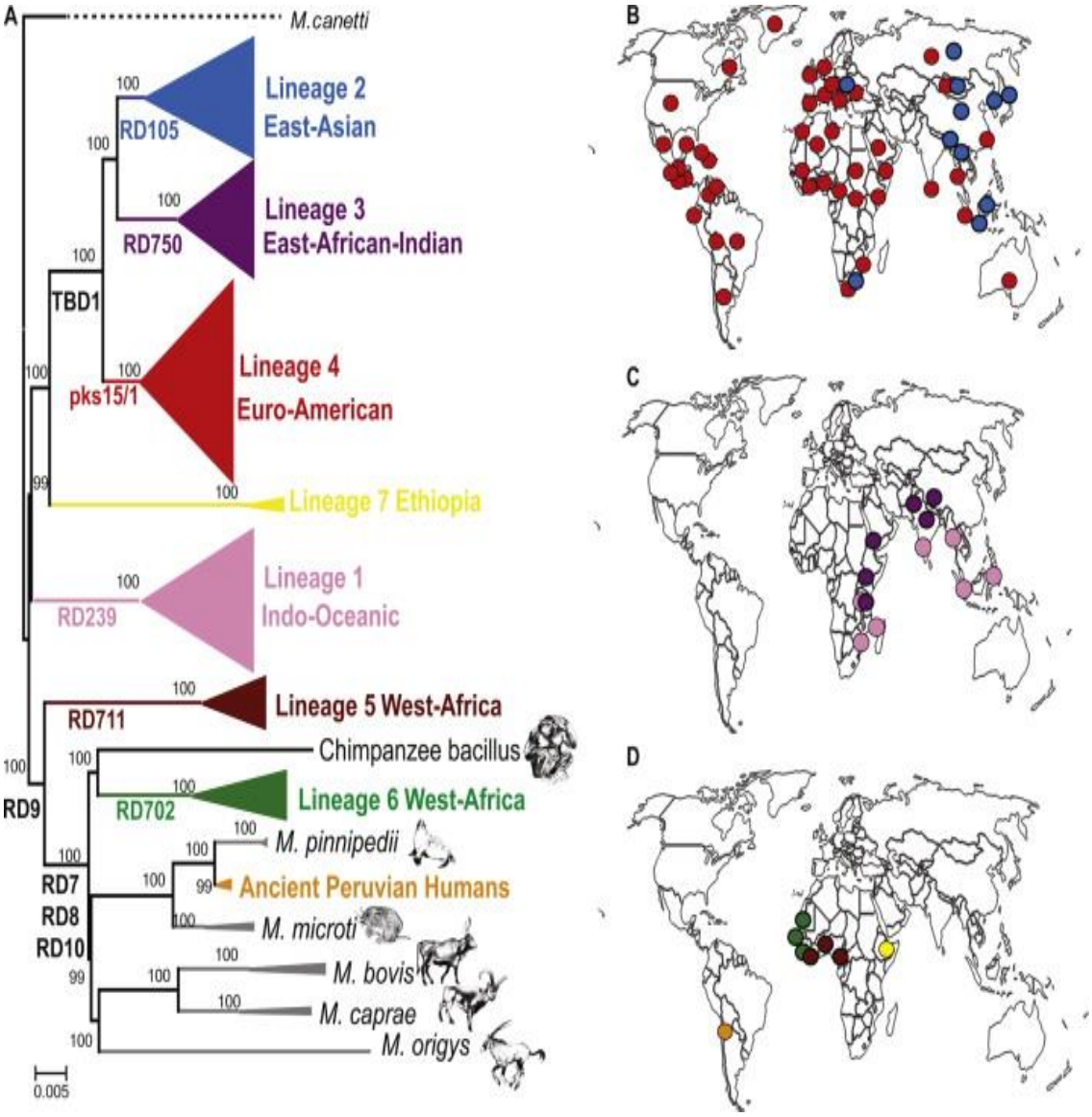

Figure 2.6 Global distribution of human-adapted *M. tuberculosis* complex (a) Maximum likelihood phylogeny modified from. (b) The most geographically widespread lineages. (c) The intermediately distributed lineages. (d)The most geographically restricted lineages (Bos *et al*., 2014).

2.4 The impact of *M. tuberculosis* Genotypes on mycobacterial phenotype

Different MTBC strains and lineages differ in their growth rates, Immunogenicity, disease severity, disease presentation, disease transmission and also differ in their gene expression and metabolic profiles (Gehre *et al.,* 2013b; Rose *et al.,* 2013). Recently, Portevin *et al* reported significant differences in mycolic acid profiles between different MTBC strains and lineages (Portevin, *et al.,* 2014). Mycolic acids constitute is the most abundant cell wall lipid in MTBC, and play an important role in the host immune response and help the bacteria to resist oxidative stress (Vander Beken *et al*., 2011).

Different studies reported that, there is variation in virulence and immunogenicity between MTBC strains. The virulence of the MTBC is directly linked to transmission, unlike many other pathogens where transmission occurs independently of disease virulence. There is a direct link between virulence and transmission in TB infection which is different from many other pathogens (Ebert and Bull, 2003), because in TB latently infected individuals are considered as non-infectious (Barry *et al.*,,2009) and Individuals harboring the largest numbers of acid-fast bacilli in their sputum are considered the most infectious (Behr *et al.*,1999). Virulence in TB is the general result of, the ability of the bacteria to survive in face of the host immune responses, their capacity to cause lung damage, to survive the aerosolization process outside the host, successful transmission and infect a new host.

Different studies have found increased in MTBC virulence associated with reduced and delayed inflammatory responses. For example, studies in different infection models have found that strain NH878 (belongs to Lineage 2/Beijing) constantly associated with a delayed inflammatory immune response and increased virulence (Subbian *et al.,* 2013). Wang *et al*., reported that Lineage 2/Beijing strains commonly induced lower levels of TNF-α, IL-6, IL-10 and GRO-α compared to strain H37Rv in monocyte-derived macrophages and dendritic cells (Wang *et al*., 2010). The modern Lineages 2, 3 and 4, showed a lower early inflammatory response compared to Lineages1 and 6 (Chen *et al*., 2014). This modern MTBC strains are more globally wide-spread and more virulence than other lineages. Hence, the observation that "modern" strains are associated with a delayed inflammatory response (i.e. higher virulence) might be linked to the global success of these strains.

MTBC lineage variation has an effect on disease severity. Study showed that TB patients from Tanzania infected with "modern" Lineage 4 strains showed more α1-acid glycoprotein and C reactive protein, higher neutrophils counts, and a lower body mass index than those infected with Lineage 1 (Stavrum *et al.,* 2014). The higher virulence of the modern Lineage 2 induced more rapid weight loss, and led to a higher bacterial load in liver, spleen and lymph nodes (Via *et al.,* 2013) and other several studies have found that Lineage 2 associated with relapse, treatment failure, and fever early during treatment (Huyen *et al.,* 2013). This implies that, strain fitness related to the bacterial pathogenicity and transmission.

Epidemiological studies have supported that strains from "modern" lineages are more transmissible than other MTBC strains. Buu *et al.,* reported higher genotypic clustering of Lineage 2 compared Lineage 1 in Vietnam (Buu *et al.*, 2012). Similarly,

other studies in various settings have reported a higher fitness of Lineage 2/Beijing strains reflected by increases in their frequency over time (van der spuy *et al*., 2009). In some cases, the increase of Lineage 2 was associated with drug resistance (Buu *et al.*, 2012).

Molecular epidemiological studies in recent years have discovered increasing evidence that some drug-resistant patterns are not equally occurred among the family members of *M. tuberculosis* (Hillemann *et al*. 2005). In Vietnam, resistance to isoniazid or streptomycin was found to more likely occur in Beijing genotype strains (Anh *et al.*, 2000). In Russia, resistance to streptomycin, rifampin, or ethambutol was found to be associated with Beijing strains (Toungoussova *et al.*, 2002). In the UK, resistance to streptomycin was associated with both Beijing and Delhi strains (Baker *et al.*, 2004). An association between resistance to isoniazid or ethambutol and Beijing genotype was also demonstrated in Taiwan (Jou *et al.*, 2005).

Moreover, by correlating resistance-conferring mutations with the genotypic lineages of *M. tuberculosis*, study found that the streptomycin resistance conferring mutation rpsLA128G was positively associated with Beijing strains, but inversely associated with Haarlem strains; the isoniazid resistance-conferring mutation *kat*G was associated with Delhi strains, and the *inh*A C15T promoter mutation positively associated with East-African-Indian (EAI) strains (Baker *et al*. 2004). In a very recent study in Germany, found that the isoniazid resistance-conferring mutation *kat*G S315T and the rifampin resistance-conferring mutation *rpo*B S531L were associated with Beijing strains (Hillemann *et al*. 2005). These findings suggest that the resistance-conferring mutations in *M. tuberculosis* may not independently and randomly occur in different genotype strains but genotype-related.

2.5 Anti-TB Drug Resistance

Drug resistance is defined as a decrease in susceptibility of an isolate to a sufficient degree for the drugs (WHO, 1997). When one percent or more of an isolates are resistant to an anti-TB drug, therapeutic success is less likely to occur and the isolate is then considered resistant to the drug.

Drug resistance *M .tuberculosis* is classified as primary and acquired resistance. primary resistance occurs when a patient is infected with a resistant strain whereas, acquired resistance occurs as a result of unsuccessful anti-tuberculosis treatment due to a number of man-made factors, such as improper medical management, inappropriate prescription, or poor adherence of patients to treatment, in this condition drug resistant mutants are selected as a result of ineffective treatment (Johnson *et al.,* 2009).The level of primary resistance in a community is an indicator of transmission of the disease within the community and the level of acquired resistance reflects the performance of on-going tuberculosis control program and success of treatment (WHO, 1997;WHO, 2000). There are different types of drug resistance TB.

Mono-resistant TB: TB in patients whose infecting isolates of *M. tuberculosis* are confirmed to be resistant in vitro to only one first-line anti-TB drug.

Poly-resistant TB: TB in patients whose infecting isolates are resistant in vitro to more than one first-line drug, other than Isoniazid and Rifampicin

Multi-drug resistant TB (MDR): is active TB involving *M. tuberculosis* organism that are resistant to Isoniazid and Rifampicin, the two most powerful anti-TB agents (Telles *et al*., 2002).

Extensive-drug resistant TB (XDR): is defined as resistance to at least Rifampicin and Isoniazid, in addition to any Fluoroquinolone, and to at least one of the three injectable drugs used in anti-TB treatment: Capreomycin, kanamycin and Amikacin.

2.6 Anti-tuberculosis drugs

2.6.1 First line anti-tuberculosis drugs

The anti TB -drug regimens are grouped into two groups called first line and second line drugs. The first line drug recommended for the treatment of new cases of pulmonary TB consists of two months intensive treatment with isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) administration, followed by four months treatment with INH and RIF (Khosravi1 *et al.,* 2006). This treatment is usually effective against wild MTB strains that have never been exposed to anti-TB drugs for more than 30 days (Churchyard *et al*., 2000).

There are two categories for tuberculosis cases (category I and category II) (WHO, 2015).

Category I: Consists mainly of new, smear-positive tuberculosis cases, but includes new parenchymal lesions, and new cases with severe extra pulmonary tuberculosis (disseminated, meningeal, pericardial, peritoneal, bilateral pleural, spinal, intestinal and genito-urinary). A new case is defined as a patient who has never previously been treated for tuberculosis or who has received treatment for less than one month (WHO, 2015).

Category II: smear-positive cases who have already received treatment for at least one month in the past and who need to receive re-treatment (WHO, 2015). Among these patients three groups can be distinguished in:

"Relapses" – patients who have been treated and declared cured, but whose smear examinations are once again positive.

"Failures" – patients whose smear examinations have remained positive or have once again become positive five or more months after starting treatment.

"Return after interruption" – patients who return to the health center smear-positive after interrupting treatment for more than two consecutive months.

In Ethiopia, the treatment regimens for category I tuberculosis case is 2 months RIF-INH-EMB-PZA and 4 months of RIF and INH. For Category II 2 months STM ,RIF,INH,EMB and PZA; 1month RIF,INH,EMB, PZA and 5 months EMB, RIF and INH(FMOH, 2012).

Rifampicin (RIF): In combination with isoniazid rifampicin forms the backbone of short-course chemotherapy. It is included in a select category of agents, which retain activity against slow-growing, and even non-replicating, *Mtb* bacilli (Telenti *et al*., 1997a). This type of antibiotic effect is important for TB, where low metabolic activity and non-replication are considered key factors in persistent *Mtb* infection. The role of RIF in sterilizing slowly metabolizing bacillary populations is a major factor in the continued reliance of public health programmes (Rattan *et al*., 1998; Ramaswamy and Musser, 1998). The mechanism of action of rifampicin is inhibition of RNA synthesis.

Isoniazid (INH): - This drug kills the great bulk of bacteria, rapidly rendering the patient non-infectious within days of starting treatment. It inhibits cell wall synthesis.

INH and RIF are by far the most important drugs in the treatment of TB, with these two drugs alone for nine months will provide cure in 95% of cases (Devis, 1998). However, patients should not start the two drugs alone in case resistance is present to one of them. In practice, the new patients should be started on INH and RIF plus PZA or EMB.

Pyrazinamide (PZA): Pyrazinamide used to allow the length of treatment to be reduced from 9 to 6 months. One key characteristic of pyrazinamide is its ability to inhibit semi-dormant bacilli residing in acidic environments. Pyrazinamide is activated to pyrazinoic acid and the mechanism of action for PZA is believed to be inhibition of fatty acid synthase I (FASI), an enzyme involved in fatty acid biosynthesis (Zimhony *et al.*, 2000). Another mechanism suggested is cytoplasmic acidification and has effect on membrane energy metabolism (Zhang, 2005). It is active against tubercle bacilli at acid pH.

Streptomycin (STM): antibiotic primarily interferes with protein synthesis by inhibiting initiation of mRNA translation through binding to ribosomal protein or 16S rRNA (Telenti *et al*., 1993), facilitating misreading of the genetic code and damaging the cell membrane (Zhang, 2005).

Ethambutol (EMB): is one of the first-line anti-TB drug which inhibits cell wall synthesis. EMB inhibits arabinosyltransferase, an enzyme important for cell wall synthesis (Takayama and Kilburn, 1989). It acts on rapidly proliferating organisms and less effective drug compared to other drugs. All first-line anti-TB drugs are said to be bacteriocidal except Ethambutol which is bacteriostatic (Zhang, 2005).

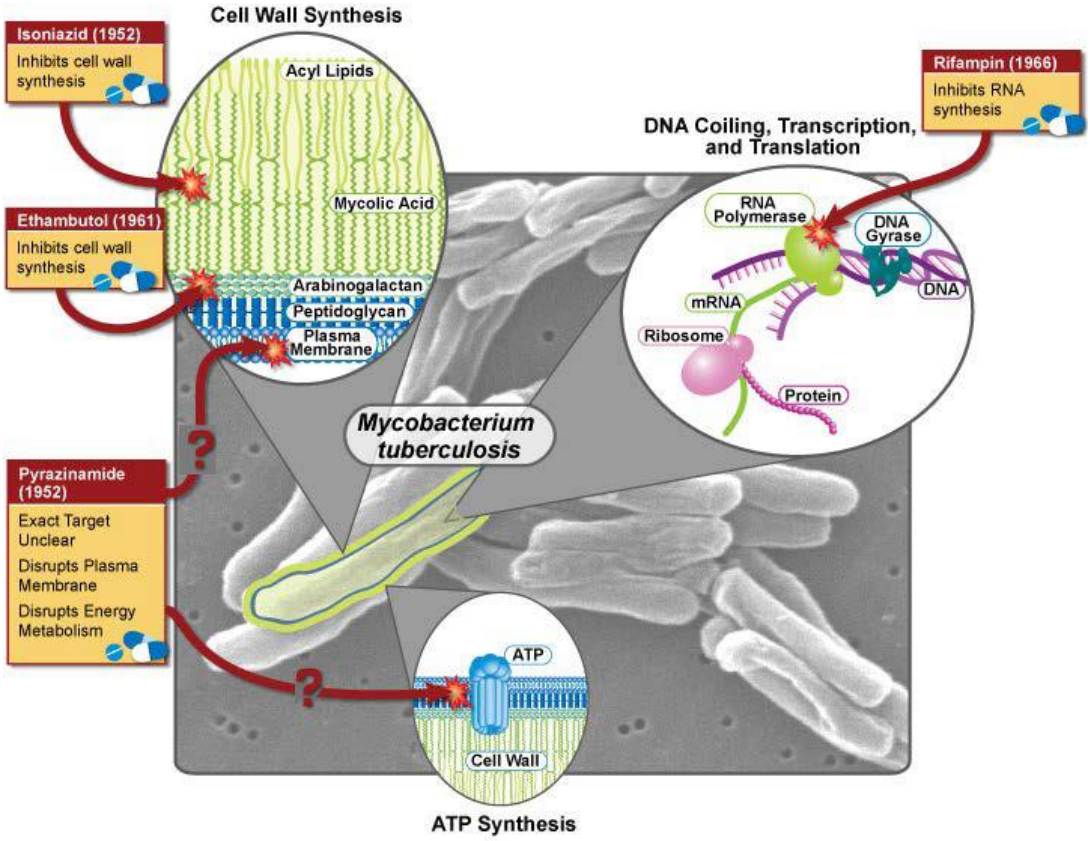

Figure 2.7 TB Primary Drugs and Mechanisms of Action. (*National Institute of Allergy and Infectious Diseases (NIAID))*

2.6.2 Second line anti-tuberculosis drugs

Second line anti-tuberculosis drugs are used in the treatment of MDR-TB. According to the WHO report (WHO, 2006) amino glycosides (kanamycin and amikacin), polypeptides (capreomycin), fluoroquinolones (ciprofloxacin, ofloxacin and levofloxacin), D-cycloserine, *P*aminosalicylic acid and thionamides (ethionamide) are classified as the second-line anti TB drugs. Some of these second-line drugs are toxic and can lead to negative side-effects (WHO, 2001). They must be taken for up to two years in order to eradicate the infection. The costs of curing MDR-TB can be staggering as much as many times that of regular treatment. For effective treatment of the MDR-TB, a detailed history of possible TB drugs which the patient has had before must be obtained. The patient should then be put on at least three drugs and preferably four drugs to which they have not had previous exposure (Lemuse *et al.*, 2004). INH and RIF should also be given at the start as if the strain, which the patient is infected with, is sensitive to these drugs time will be gained by giving them from the start. For instance, if the patient has had previous treatment with INH, RIF, and PZA and EMB, INH and RIF should be given, and injectable drug such as amikacin (AM), at least one of the old drugs such as ethionamide (ETO) and one of the new drugs such as ciprofloxacin (CFX) are recommended. D-cycloserine(DCS) could be used as a fourth drug if required. Thus, the patient is treated with six drugs. The risk of adding a single drug to a regimen already being given will therefore be avoided (WHO, 2001).

2.7 Molecular Mechanisms of the emergence of drug resistance

In order to control the drug resistance epidemic it is necessary to get information how *M. tuberculosis* develops drug resistance. Such knowledge will help us to understand how to prevent the occurrence of drug resistance as well as identifying genes associated with drug resistance of new drugs.

Other bacteria may transmit antibiotic resistance determinants through transmissible genetic elements, transposons, integrons, and plasmids, by transduction or transformation (Gillespie, 2002). This option is not available for *M. tuberculosis,* so resistance can only occur through chromosomal mutation although rarely movement of mobile genetic elements, such as the IS*6110,* has been associated with new resistance emerging through the inactivation of critical genes. Resistance of *M. tuberculosis* to anti-TB drugs is a man-made amplification of a natural phenomenon. Wild strains of *M. tuberculosis* that have never been exposed to anti-TB drugs are almost never resistant (Bottger, 1999). During bacterial multiplication, resistant bacilli evolve through spontaneous mutation and with defined frequency (Almedia and palomino, 2011). For instance, mutation that results in INH resistance to *M. tuberculosis* occurs at a rate of 10^{-7} to 10^{-9} per cell division and leads to an estimated resistance of 1 in 10^6 bacilli in drug-free environment (Gillespie, 2002). However, these resistant organisms (mutants) are diluted within the majority of drug-susceptible mycobacteria since bacillary populations greater than10^7 are common in the lung of TB patients (Almedia and palomino, 2011).

Mutation amplified by inappropriate treatment the patient to TB infection. The presence of a single antimicrobial drug, to which mutation has been developed, provides the selective pressure that helps resistant organisms to predominate. This

process could especially be common in situations where there is a large load of bacilli such as in cavities. Exposure to a single anti-TB drug due to irregular intake, poor drug quality, inappropriate prescription and/or poor adherence to treatment could result in functional mono therapy. This will suppress the growth of bacilli susceptible to that drug but permits the multiplication of drug resistant organisms and results in acquired drug resistance (secondary drug resistance). Subsequent transmission of such bacilli to other persons may lead to disease which is drug-resistant from the outset, initial drug resistance (primary drug resistance). When this process is repeated through the same process, multiple drug or poly drug resistance will be developed.

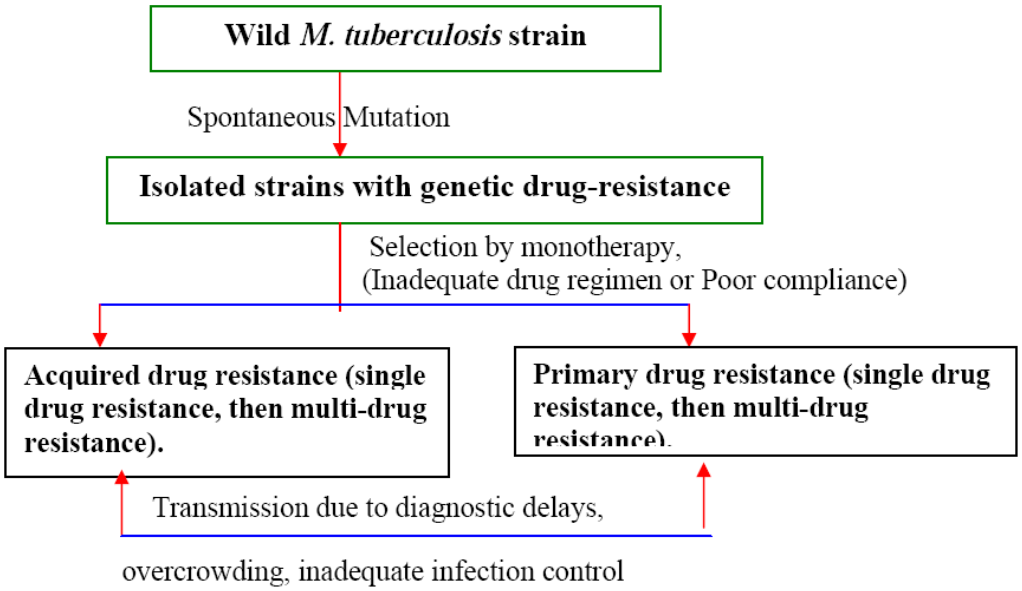

Figure 2.8 Development and spread of single drug and multi drug resistance tuberculosis (WHO, 2000).

2.8 Genes responsible for resistance

The primary mechanism for acquiring resistance in *M.tb* is the accumulation of point mutations in gene coding for drug targets or drug-converting enzymes (Irfan *et al.,* 2007)**.** There are different genes which are responsible for the occurrence of the mutations in different target of TB drugs. Generally, resistance to first line anti-TB drugs has been linked to mutations in at least 11 genes *katG, inhA, ahpC, kasA, mabA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STM resistance (Xue-qiong *et al*., 2005).

Rifampicin resistance regions: - RIF resistance is a results of single-nucleotide substitution mutations in a small region of *rpoB* gene, the gene encoding the β-subunit of the DNA-dependent RNA polymerase (RNAP). The majority of RIF resistance mutations occur in an 81 bp region of the so-called RIF resistance determining region (RRDR) (Rattan *et al*., 1998; Ramaswamy and Musser,1998) though some mutations have been identified in other regions of *rpoB* as well (Zhang and Telenti., 2000).

Of the 12 amino acid residues that surround the RIF binding pocket, 11 have been associated with RIF resistance mutations (Campbell *et al*., 2001). This resistance occurs on *rpoB* gene between codons 507-533 with the most common changes in codons Ser 531 Leu, His 526 Tyr and Asp 516 Val. These changes occur in more than 70% of RIF resistant isolates (Herrera *et al*., 2003).

Isoniazid resistance regions: - it is a pro-drug requiring activation by the catalase/peroxidase, enzyme encoded by *katG*. Activated isoniazid interferes with the synthesis of essential mycolic acids by inhibiting NADH-dependent enoyl-ACP reductase, which is encoded by *inhA*. Resistance to isoniazid is caused by mutations in several genes, including *katG*, *ahpC*, *inhA*, *kasA* and *ndh* (Ramaswamy and

Musser, 1998). Different studies found that mutations in *katG*, *inhA* and *ahpC* were most strongly associated with isoniazid resistance, while mutations in *kasA* were not associated with resistance (Suarez *et al.,* 2009). Above all mutation the most common one for INH resistance is mutation in *katG*. The *KatG* is important for the virulence of *M. tuberculosis* due to its role in oxidative stress management.

More than a hundred mutations in *katG* have been reported which decrease or total loss of catalase/peroxidase activity associated with isoniazid resistance.(Ramaswamy and Musser, 1998). Mutations in *inhA* cause not only resistance to isoniazid, but also resistance to the structurally related second-line drug ethionamide. The most common *inhA* mutation occurs in its promoter region (−15C→T) and it has been found more frequently associated with mono-resistant strains ((Ramaswamy and Musser, 1998).

In *M. tuberculosis, ahpC* codes for an alkyl hydro peroxidase reductase that is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. It was initially proposed that mutations in the promoter of *ahpC* could be used as surrogate markers for the detection of isoniazid resistance. However, several other studies showed that an increase in the expression of *ahpC* seems to be more a compensatory mutation for the loss of catalase/peroxidase activity rather than the basis for isoniazid resistance (Rattan *et al.,* 2014).

Pyrazinamide resistance regions: - Pyrazinamide is activated to an active form, pyrazinoic acid, by pyrazinamidase, an enzyme encoded by *pnc*A. Mutations in the *pncA* gene correlate well with phenotypic resistance to PZA (70%). However, PZA resistant isolates without *pncA* mutations were also observed suggesting that another mechanism may be involved in conferring PZA resistance in these isolates (Johnson *et al.,* 2009).

Streptomycin resistance region: - Resistance to STM is mainly due to mutations in the *rps*L locus encoding the S12 ribosomal protein (Ramaswamy and Musser, 1998). Approximately 60% of Streptomycin resistant clinical isolates show *rps*L mutation and about 10% of resistant strains have mutations in 16S ribosomal RNA which is encoded by *rrs* gene ((Ramaswamy and Musser, 1998).

Ethambutol resistance region: - The major mechanism of acquisition of resistance to the drug is associated with point mutations in the *emb*CAB operon (Telenti *et al*., 1997b). This operon is composed of three organized genes encoding different arabinosyl transferases that are involved in cell wall synthesis (Ramaswamy and Musser, 1998).

Furthermore mutations in different genes are observed against different second line drugs. The quinolone resistance-determining region (QRDR) is a conserved region in the *gyrA* (320bp) and *gyrB* (375bp) genes (Ginsburg *et al*., 2003), which is the point of interaction of FQ and gyrase (Ginsburg *et al*., 2003). Mutations in the promoter of the *inhA* gene are associated with resistance to INH and ETO (Morlock *et al*., 2003) .Over expression of *alr* cause DCS resistance, trans version in the *alr* promoter may lead to the over expression of *alr* (Feng and Barletta, 2003). An association between mutations in the *rrs* gene that encodes the 16S rRNA and resistance to CAP and KAN has been reported and mutation of the *tlyA*gene encoding a putative rRNA methyl transferase confers resistance to CAP (Morlock *et al*., 2003).

Table 2.2 Molecular genetic target of Anti TB drug resistance and their rate of mutation.

Drugs	Year of Introduction	Molecular target	Target gene(s)	Mutation rate*	Wild type resistance
INH	1952	Mycolic acid synthesis	*kat* G, *inh* A, *ahp* C, *kasA*	10^{-8}	1 in 10^{6}
RMP	1965	RNA polymerase	*rpo* B	10^{-10}	1 in10^{8}
PZA	1970	Not known	*pnc* A	10^{-3}	1in 10^{6}
STM	1944	Ribosomal protein	*rps* L *rrs*	10^{-8}	1 in 10^{7}
EMB	1968	Cell wall polysaccharides	*Emb* A, B & C	10^{-7}	1in 10^{5}

**Rate of mutation per cell division at the gene(s) responsible for drug resistance.* (WHO, 1997)

2.9 Methods for the Detection of Drug Resistance TB

2.9.1 Conventional methods: is a method based on the cultivation of *M. tuberculosis* from clinical samples. The followings are major conventional methods for anti-tuberculosis drug susceptibility testing (WHO, 2001).

a) The absolute concentration method: In this method, media containing several consecutive two-fold dilutions of each drug are used and resistance is indicated by the lowest concentration of the drug which will inhibit growth.

b) The resistance ratio (RR) method: This is defined as the minimum inhibitory concentration (MIC) of the test strain divided by the MIC of the standard susceptible strain H37Rv in the same set of tests. A RR of 2 or less defines drug susceptibility, a RR of 4 is considered low level or intermediate drug resistance while a RR of 8 or more is considered evidence of high drug resistance (WHO, 2001).

c) The proportional method: This is the most commonly used method where the number of colonies growing on drug-containing medium and the number of colonies growing on drug-free medium counted. From these bacterial colony counts, the proportion of mutants resistant to the drug concentration tested can be determined and expressed as a percentage of the total number of viable colony forming units in the population (Heifets and Cangelosi, 1999). Resistance is defined as more growth (above 1%) in drug containing media as compared to drug free controls. It is the only method in which the validity of critical drug concentrations and drug resistance proportions has been correlated to bacteriological as well as clinical criteria (Laszlo *et al.,* 2002).

d) Bactec MGIT (mycobacteria growth indicator tube) 960:-is Rapid phenotypic method uses an oxygen-quenching fluorescent sensor technology in conjunction with unique algorithms to determine positivity of the culture tubes. BACTEC MGIT 960 medium is a tube containing Middlebrook 7H9 Broth, which supports the growth and detection of mycobacteria (Telles, *et al*., 2002).

The tube also contains a fluorescent compound embedded in silicone on the bottom of a 16 x 100 mm round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. Fluorescence can be recorded by automated instruments such as Becton Dickinson's BACTEC MGIT 960 System.

All of the above conventional DST methods which are standardized and are widely used throughout the world to measure drug resistance of *M. tuberculosis*. It is reported that these methods are highly reliable and reproducible, and that the results do not differ according to the method used (WH0, 2006). The drawbacks of these culture-based methods is the time they take: *M. tuberculosis* detection takes an average of 3 weeks under optimal conditions, and drug susceptibility testing takes an additional 3 to 4 weeks, requiring a minimum of 6 weeks for DST result which leads to prolonged infectiousness (Heifets and Cangelosi, 1999). In the case of liquid media there is an average reduction of 15 days in total (from culture up to DST). Moreover, these DST methods are labor intensive and costly. Therefore, more advanced, robust and rapid technologies are needed to strengthen laboratory capacity for rapid and accurate diagnosis of drug-resistant strains of *M. tuberculosis* which is important to control transmission of TB.

2.9.2 Molecular methods for detecting drug resistance

Advances in the field of molecular biology and progress in the understanding of the molecular basis of dug resistance in *M. tuberculosis* have provided new tools for its rapid diagnosis by molecular methods (Takiff, 2000). *M. tuberculosis* is a very slow growing organism which needs about four or more weeks for culturing therefore, for the rapid detection of mutations in resistance causing genes molecular methods are preferable. Molecular methods can also be used on specimens that are not suitable for growth–based assays, such as specimens containing non-viable bacteria (killed by heat or chemical inactivation during drug therapy), specimens highly contaminated with non-mycobacterial flora, or specimens with mixed TB and non-tuberculous

mycobacteria (NTM). These assays do not require viable bacteria, therefore specimen transport conditions do not affect test outcomes, unlike culture, and specimens can be shipped by regular mail (Viedma *et al*., 2003).

The techniques that are applied for the genotypic analysis of resistance in *M. tb* can be sequencing or probe hybridization-based assays (Viedma *et al*., 2003). The probe hybridization-based assays are the most common and commercially available assay.

Sequencing: - is the most direct way of defining resistance genotypically. It requires the amplification of a region which includes the Codons involved in the resistance, and these amplicons are sequenced to assign to the presence/absence of a specific mutation (Victor *et al*., 2002). Sequencing provides more detailed information and detection of specific mutations. It enables correlation between specific mutations and phenotypic DST. Its limitation is that it is expensive and requires expertise (Victor *et al*., 2002).

The probe hybridization-based assays: - based on the hybridization of PCR products from patient specimens to specific probes for wild-type and mutant alleles of genes involved in drug resistance and have shown high specificity and sensitivity. These methods are started by PCR amplification of target genes associated with drug resistance in M. *tuberculosis,* followed by selective hybridization under stringent conditions with allele-specific labeled probes. If the two strands are different no hybridization can occur (Victor *et al*., 1999; Mokrousov *et al*., 2004). These methods include the molecular beacons (GeneXpert) and Line probe essays (LiPA).

GeneXpert MTB/RIF Assay

The GeneXpert MTB/RIF assay detects *M.tb* DNA in sputum and other sample types. Five probes are used to differentiate between the conserved wild-type sequence and mutations in the RRDR that are associated with RIF-Resistance. It identifies mutations in the *rpo*B gene, which are associated with resistance to rifampicin. In this method, *M. tuberculosis* DNA sample is prepared and transferred into the MTB/RIF cartridge and entered into the GeneXpert instrument. By starting the test on the system software, the GeneXpert automates all following steps, including sample work-up, nucleic acid amplification, detection of the target sequence and result interpretation. The primers in the GeneXpert MTB/RIF assay amplify a portion of the *rpo*B 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 are associated with resistance to RIF. The GeneXpert MTB/RIF assay as an entirely self-contained test with quality control of the various steps included; so it is not necessary to perform testing of quality controls with every batch of tested specimens.

Line Probe Assays (LiPA)

There are currently two commercially available Line probe assay techniques: the Line Probe Assay (INNO-LiPA Rif TB Assay; Innogenetics, Ghent, Belgium) for the detection of rifampicin resistance and the GenoType MTBDR assay (Hain Life sciences, Nehren, Germany) for the simultaneous detection of isoniazid and rifampicin resistance (Victor *et al*., 1999; Mokrousov *et al*., 2004).

a) INNOLiPA Rif: - is TB kit simultaneously detects the *M. tuberculosis* complex and the presence of mutations in the *rpoB* gene associated with resistance to

rifampicin which is considered a marker for MDR-TB strains. The strip contains 5 probes for detection of sensitive genotypes (S1-S5) and 4 probes for detection of resistance genotypes (R2, R4a, R4b and R5) (Makinen *et al*, 2006). Rifampicin resistance is indicated by the absence of one or more sensitive probes, possible accompanied by the appearance of one or more mutant probes (Cooksey et *al.,* 1997; Rossau *et al.,* 1999).

b) The GenoType MTBDR*plus* assay (version 1):- is able to detect mutations in the *rpoB* gene for RIF resistance, and the most frequent mutation at codon 315 of the *katG* gene for INH resistance, either in isolates or clinical specimens (Watterson *et al*.,1998). The specificity and sensitivity of the assay for RIF resistance were nearly 100%; for INH-resistance, despite a high specificity (approximately 100%), the sensitivity of the test ranged from 70% to 90%, depending on the prevalence of the particular mutation at the *kat*G locus (Hilleman *et al*., 2007).

GenoTypeMTBDR*plus* assay (version 2):- an advanced version of the assay, includes probes for the identification of other mutations in the hotspot region of the *rpoB* gene for RIF resistance, and probes to detect mutations in the promoter region of the *inhA* gene involved in INH resistance. These improvements facilitate the detection of another INH-resistant case, with an enhancement in rapid MDR-TB diagnosis. This version has the ability to detect a broader variety of *rpo*B gene mutations and has added probes for wild type and mutations in *inh*A in addition to *kat*G. By covering mutations in the regulatory region of *inh*A, additional INH-resistant strains have been detected. In fact, the identification of mutations in the promoter region of *inh*A has increased sensitivity for molecular detection of INH resistance up to 31.4% as compared to detecting only mutations at codon 315 in *kat*G.

Some of the limitation of Line Probe Assays is detection of silent mutations may result in false prediction of resistance, not all mutations are associated with phenotypic drug resistance because mutation could be "silent" no change in amino acid. Such mutation may not cause significant change in protein expression, structure, or function, as with other genotypic methods the sensitivity of the test depends on the amount of DNA present in the sample and also the presence of inhibitors could cause false-negative results, this molecular test may not include probes for all possible mutations (Hirano *et al*.,1999). Most molecular tests (including the LiPAs, molecular beacons and real time PCR) only interrogate the 81 base-pair hot spot of *rpo*B and the 315 codon of *kat*G, while resistance-associated mutations may occur in other parts of these genes. Furthermore, molecular mechanisms are not fully identified and understood for all drugs, and therefore mutations in known targets may not be associated with phenotypic drug resistance in all cases (e.g. INH, aminoglycosides, fluoroquionolones etc.). The line probe assays are accurate and useful for rapid detection of drug resistance directly in clinical specimens (Watterson *et al*., 1998). In general, line probe assays are expensive and require sophisticated laboratory infrastructure. Their role and utility in low income, high-burden countries need to be evaluated in field studies.

2.10 Preventing Drug-Resistant TB

The most important way to prevent the spread of drug-resistant TB is to take all TB drugs exactly as prescribed by the health care provider. No doses should be missed and treatment should not be stopped early. People receiving treatment for TB disease should tell their health care provider if they are having trouble taking the drugs.

Health care providers can help prevent drug-resistant TB by quickly diagnosing cases, following recommended treatment guidelines, monitoring patients' response to treatment, and making sure therapy is completed.

Another way to prevent getting drug-resistant TB is to avoid exposure to known drug-resistant TB patients in closed or crowded places such as hospitals, prisons, or homeless shelters. People who work in hospitals or health-care settings where TB patients are likely to be seen should consult infection control or occupational health experts.

CHAPTER THREE

3. Materials and Methods

3.1 Study area

This study was performed at three different sites in central Ethiopia. These sites were Woliso and Atat towns and their surroundings in the southwest of Addis Ababa at a distance of 114 km and 187 km, respectively. The third site was Fiche town and its surrounding in the northwest of Addis Ababa at 115km. Sample collection was performed at hospitals located at these three sites, namely, St. Lukas, Atat and Fiche hospitals located at Woliso, Atat and Fiche towns, respectively.

Woliso is located in West Shewa, Waliso zone; the town has a population of 54,248 people (28,620 males and 25,628 females). Woliso, a rapidly growing town, has one hospital (St. Luke Hospital), one health center (Woliso Health Center). The height of Woliso above sea level is about 1900 meter with annual rain fall of 1200mL, has a latitude and longitude of 8°32′N 37°58′E and temperature of 18-27 ^{0}c. At the moment the town of Woliso has area coverage of 2,225.25 hectare. St. Luka Hospital is found in this town which is the only Hospital which provides various health services for woliso society the surrounding areas or woredas.

Attat located at the South west of Addis Ababa along the Jimma Road in the Southern Region of Ethiopia, Gurage Zone, and Cheha Woreda. Gurage Zone is found Ethiopian Southern Nations, Nationalities and Peoples Region (SNNPR). It is a densely populated, rural area. Attat Hospital is found in this zone which is the only Hospital which provides various health services for cheha Worda society and neighboring woredas of Gurage Zone.

Fiche is located in the North Shewa Zone of Oromiya region, at northwest of Addis Ababa. The town has a population of 49,129 (22,820 males and 26,309 females). Fiche has better infrastructure (schools and health institutions) than other towns in North Shewa. It has one hospital, and one health center. Fiche has a latitude and longitude of 9°48′N 38°44′E and an elevation between 2,738 and 2,782 metres above sea level. Fiche Hospital is found in this town which provides various health services for fiche society and neighboring woredas.

Sputum samples were collected from smear positive TB cases visiting these three hospitals. These study areas were selected as representative of central Ethiopia using convenient sampling based on prier contact to the established data.

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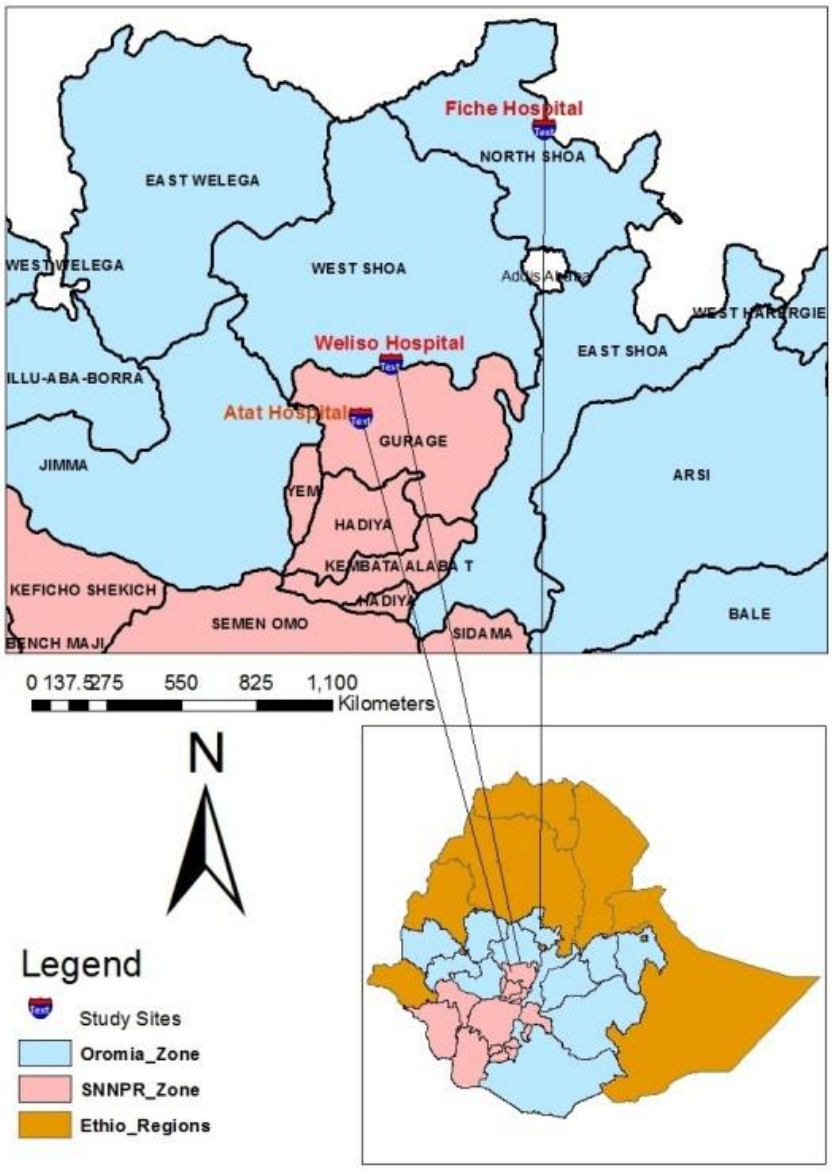

Figure 3.1: Location of south west and north part of central Ethiopia (Weliso Fiche ,Atat and their surroundings area).

3.2. Study design and study subjects

This study was a health institutional based cross sectional study that was conducted on newly and previously treated adults (>18 years old) TB cases. A total of 338 sputum samples were collected from smear positive TB cases visiting three hospitals in central Ethiopia, between October 2012 and September 2013. The physicians in charge of diagnosis collected patients' demographic data.

3.3. Sample Size

The sample size was determined by taking the prevalence of rifampicin resistance of 1.6% from a previous study (WHO, 2015), desired precision of 1%, a 95% confidence level and a nonresponse rate of 20%. The sample size was calculated based on the sampling method recommended by WHO for drug resistance survey in tuberculosis (WHO, 2015 b). The final sample size calculated was 338. The total number of new smear positive PTB patients registered in each study site in the year 2012 was 243, 102, and 225 in Woliso, Atat, and Fiche, respectively. Thus, in total 570 new smear positive PTB patients were registered in the same year. Based on this patient load, the sample size proportionally allocated for each site was 145, 60, and 133 for Woliso, Atat, and Fiche, respectively.

3.4 Sample processing and Culture

Morning and spot sputum samples were collected and processed for culture following the WHO Guideline (WHO, 1998). Sputum samples were cultured at Aklilu Lemma Institute of Pathobiology TB Laboratory. Briefly, equal volume of 4% NaOH was

mixed with sputum sample and the mixture was centrifuged at 3000 rpm for 15 minutes at room temperature. After decanting the supernatant the sediment was neutralized with 2 N HCl using phenol red as an indicator. Neutralization was achieved when the color of the solution was changed from purple to yellow. Thereafter, 100 µl of the suspension was inoculated onto two sterile Löwenstein – Jensen medium (LJ) medium slopes (which were enriched with either pyruvate or glycerol). The inoculated media were then incubated at 37ºC in slanted position for one week and upright position for four to five weeks. The growth of the bacteria was read every week until the 8th week of culture.

3.5. Preparation of DNA for molecular typing

Colonies were removed from the surface of LJ medium and suspended in 200 µl of sterile double distilled water. Thereafter, the colonies and water were mixed thoroughly and then, the mixture was heated at 80ºC for 1 hour in water bath. This is followed by centrifugation after which the supernatant was collected and used for amplification**.**

3.6 Region of difference (RD) 9-based polymerase chain reaction (PCR)

Identification of *M. tuberculosis* from the other members of *M. tuberculosis* complex species was done using RD9-based PCR. RD9-PCR was performed on heat-killed cells to confirm the presence or absence of RD9 using three primers namely, RD9 flankF, RD9 IntR, and RD9 flankR (Parsons *et al*., 2002). Amplification was done by standard thermo cycler (VWR Thermo cycler, UK). The PCR amplification mixture used consisted of 10 µL HotStar Taq Master Mix (Qiagen, United Kingdom), 7.1 µL distilled water, 0.3 µL of each three primers and 2 µL of DNA template (heat killed

cells), giving a total volume of 20 µL. The PCR reaction was heated at 95^{o}C for 15 minutes after which it was subjected to 35 cycles consisting of 95 ^{o}C for one minute, 55 ^{o}C for one minute, and 72 ^{o}C for one minute. Thereafter, the reaction mixture was maintained at 72 ^{o}C for 10 minutes following which the product was removed from the thermo cycler and run on agarose gel electrophoresis. For gel electrophoresis, 8 µL PCR products was mixed with 2 µL loading dye, loaded onto 1.5% agarose gel and electrophoresed at 100 V and 500 mA for 45 minutes. The gel was then visualized using a computerized Multi- Image Light Cabinet (VWR). *M. tuberculosis* H37Rv, *M. bovis* bacille Calmette-Guérin, and water were included as positive and negative controls. Interpretation of the result was based on bands of different sizes, as previously described by Parsons *et al* (Parsons *et al*., 2002).

3.7. Spoligotyping

Isolates that were positive for *M. tuberculosis* by RD9 PCR were further characterized by spoligotyping following the procedure described by Kamerbeek *et al* (Kamerbeek *et al.*, 1997) and by observing the instructions of the spoligotype kit supplier (Ocimum Bio solutions Company, Iisselstein, and the Netherlands). The direct repeat (DR) region of the isolate was amplified by PCR using oligonucleotide primers (DRa and DRb) derived from the DR sequence. The amplified biotinylated products were hybridized to a set of 43 oligonucleotides covalently bound to a membrane (Animal and Plant Health Agency, Great Britain). Bound fragments were incubated with streptavidin peroxidase conjugate and hybridizing DNA was detected by the enhanced chemiluminescence method, by exposure to X-ray film (Hyperfilm ECL, Amersham) as specified by the manufacturer's instruction. The presence and absence of spacers was visualized on the film as black and white squares, respectively. Characterized

strains of *M. bovis* and *M. tuberculosis* H37Rv were used as positive controls, whereas Qiagen distilled water (Qiagen company, Germany) was used as a negative control.

3.7.1 Use of SpolDB4 and Run TB-Lineage for the identification of strains and lineages

The results of spoligotyping were converted into octal and binary formats. These binary and octal formats of the strains were entered into query box so that the name of the strains are retrieved from the database if the spoligotype pattern of the strain in question fits the pattern that has already been registered in the SPolDB4 database (Brudey *et al.*, 2006) and at http://www.pasteur-guadeloupe. fr:8081/SITVITD emo/ (SITVIT1). If the pattern of the strain in question has not been registered prior, no name was retrieved, and hence the strain was considered as an orphan.

Two or more mycobacteria isolates sharing identical spoligotype patterns in the study were identified as clusters, whereas single spoligo pattern were considered as unique strains. Strains matching a pre-existing pattern in the SITVIT2 database were identified with the shared international type (SIT) number. An online tool Run TB-Lineage http:// tb insight. cs.rpi.edu/run_tb_lineage.html was also used to predict the major lineages using a conformal Bayesian network (CBN) analysis and sub lineage using knowledge based Bayesian network (KBBN).

3.8 MIRU-VNTR typing

MIRU-VNTR typing performed on 29 selected *M. tuberculosis* isolates at the J. Craig Venter Institute (JCVI), USA. Heat killed mycobacterial DNA were shipped to JCVI and were analyzed by the standardized 24-locus MIRU-VNTR typing as previously

described (supply *et al*., 2006). In brief, 24 MIRU-VNTR markers were amplified by PCR using the fluorescent-labeled PCR primers (Appendix) flanking each polymorphic MIRU-VNTR locus and multiplex mixtures. Each MIRU-VNTR locus was individually amplified using PCR 96-well plates. The PCRs were carried out using a PCR tetrad thermo cycler starting with an enzyme activation step of 15 minutes at 95°C, followed by 40 cycles of 1 minute at 94°C for denaturation, 1 minute at 59°C for annealing, and 1 minute 30 seconds at 72°C for extension. Thereafter, the reactions were incubated for 10 minutes at 72°C for enzyme stabilization. Positive control H37Rv and negative control sterile water were included in the study.

DNA fragments were separated by capillary electrophoresis using an automated 96-capillary ABI DNA analyzer (LabChip GXII). PCR fragments were sized, and MIRU-VNTR alleles were assigned using the GeneScan version 4.0 software (Supply *et al.* 2001 and 2006). The results are presented as a set of digits, the MIRU code, that reflect the number of MIRU sequence repeats. The MIRU-VNTR 24-loci profiles were used to classify the strains into main phylogenetic lineages by using the reference strain collection and identification tools available online at http://www.miru-vntrplus.org/MIRU/.

3.8.1 Phylogenetic analyses

The MIRU-VNTR plus online web application were used to classify the *M. tuberculosis* lineages identified in this study (http://www.miru-vntrplus.org/MIRU/). The MIRU-VNTR 24 loci profiles were uploaded, and the “similarity” search option was selected to classify lineages based on the best match with the reference strains in the database. A dendrogram was generated using the unweighted-pair group method with arithmetic averages (UPGMA).

3.9 Conventional Drug Susceptibility testing using proportional method

Drug susceptibility testing (DST) was performed for the four first line drugs, isoniazid, streptomycin, rifampicin and ethambutol, using the indirect proportion method on Löwenstein–Jensen (LJ) medium. The critical concentrations for each drug were 0.2µg/ml, 4µg/ml, 40µg/ml and 2µg/ml for INH, STM, RIF and EMB, respectively. The experiments were performed following the standard protocol (Canetti, *et al.*, 1996). Briefly, one loop full of colonies was scraped and suspended in a sterile bottle containing 5-6 glass beads. Then, 5 ml of sterile water was added and vortexed. The suspension was left for 15-30 minutes to allow large particle to settle down. The supernatant was collected and added to a second bottle containing 5 ml of sterile distilled water. A bacterial suspension in a second bottle was adjusted to match McFarland standard one. Next, 100-fold dilution (10^{-2}) and 10,000-fold dilutions (10^{-4}) were prepared. One hundred microliter of the culture suspension from 10^{-4} and 10^{-2} dilutions each were used to inoculate separately to individual control LJ medium (control), and a 100 µl culture suspension of the 10^{-2} dilution was used to inoculated onto each of the four anti-TB drug containing LJ media (INH, RIF, STM and EMB) in the aforementioned concentrations (critical concentration). The inoculated media were incubated at 37^{o}C up to 6 weeks. Bacterial growth was monitored once a week (Kent and Kubica, 1985).

The interpretation of the results was done by comparing amount of growth on control media and drug containing media. The proportion of resistance was calculated by dividing the number of grown colonies on media containing the drug multiplied by 100 and divided by the number of grown colonies on the control media (in %). A

strain was considered resistant when bacterial growth on a drug containing media was equal to or greater than 1% (Kent and Kubica, 1985).

3.10 GenoTypes MTBDR*plus* assay

The GenoType MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) was performed according to the manufacturer's instructions (Nikolayevsky *et al*., 2004). The test is based on DNA strip technology and has three steps: DNA extraction, multiplex polymerase chain reaction (PCR) amplification, and reverse hybridization. The assay screens for the absence and/or presence of wild-type (WT) and/or mutant (MUT) DNA sequences within specific regions of three genes, :the *rpoB* gene for RIF resistance, the *katG* gene for high-level INH resistance, and the *inhA* gene for low-level INH resistance. Each strip contains 27 reaction zones and the results can be obtained within a day (Zhang *et al.*, 2005). In brief, for one PCR , 10µL amplification mix A containing the 10× buffer, nucleotides, and DNA polymerase was mixed with 35µL of amplification mix B containing the MgCl2, the biotinylated primers, and dye. Then, 5 µL of *M. tuberculosis* DNA was added to the mixture, making the final volume of PCR mix to be 50 µL. The PCRs consisted of 15 minutes of denaturing at 95°C, followed by 10 cycles of 30seconds at 95°C and 120seconds at 58°C, followed by 20 additional cycles of 25seconds at 95°C, 40seconds at 53°C, and 40seconds at 70°C, with a final extension at 70°C for 8 minutes. For hybridization 20 µL of the amplification products were mixed with 20 µL of the denaturing reagent (provided with the kit) and denaturing was performed for 5 minutes in each of the plastic well. Thereafter, 1 mL of pre warmed hybridization buffer was added into each well and one strip was placed in each well. The hybridization was performed at 45°C for 30minutes, followed by two washing steps. For colorimetric detection of hybridized

amplicons, streptavidin conjugated with alkaline phosphatase was added after which a substrate buffer was added. After final washing, strips were air dried and fixed on paper. The DNA of the standard strain H37Rv and molecular grade water were used as positive and negative controls, respectively.

Interpretation of Results

Each strip consists of 27 reaction zones (bands) with six controls including conjugate control (CC), amplification control (AC), *M. tuberculosis* complex, *rpoB* locus control, *katG* locus control, and *inhA* locus controls. The remaining 21 reaction zones are wild type (WT) and mutation (MUT) reaction zones including eight *rpoB* WT and four MUT probes, one *katG* WT and two MUT probes, and two *inhA* WT and four MUT probes. Results were interpreted according to the manufacturer's instructions (Nikolayevsky *et al*., 2004). In brief, the presence of CC bands indicate the efficiency of the conjugate and substrate the presence of AC bands indicates the efficiency of DNA extraction and PCR procedures, and the presence of the *M. tuberculosis* complex band indicates that the tested bacterium belongs to the *M. tuberculosis* complex. The three respective locus control bands (*rpoB, katG and inhA*) indicate the presence of the specific gene region. The absence of the WT band is usually accompanied by the presence of MUT, which indicates resistance and the presence of all WT bands without the MUT band indicates susceptible isolate. In rare cases, lack of WT band(s) without a corresponding MUT band could be observed due to uncommon mutations in the probe region and the presence of both WT and MUT bands in the same stripe might be an indication for the presence of hetero-resistance or mixed infection.

3.11 Quality control

Reliability of the data relay on the quality parameter measured during the data generation. The sterility of the culture media was checked by incubating the whole media at 37 ^{0}C for 48 hours and 10% of it for 21 days. Known susceptible *M. tuberculosis* (H37Rv) and well characterized local laboratory resistant isolates for each drug were included as controls.

3.12 Statistical Analysis

The statistical analysis was performed using STATA software version 12 and using SPSS version 20 statistical package e software. Descriptive analysis, frequencies, and odds ratios (OR) with 95% confidence interval were calculated. Categorical data were compared using chi-square test or using fisher exact test, when expected cell frequencies were smaller than 5. In order to determine independent risk factors, OR and 95% confidence intervals (CI) were calculated using logistic regression analysis. In the logistic regression model demographic variables, previous treatment history, and drug resistance were include as confounding variables. Results with p-values less than 0.05 were considered as being statistically significant.

3.13 Ethical Considerations

Ethical clearance was obtained from Ethical Review Board of Natural Science, Addis Ababa University, Ethiopia (Ref. No. CNSDO/379/07/15). In addition, the purpose of the study was explained to all enrolled subjects in simple terms, and written informed consents were obtained from each of the study participants.

CHAPTER FOUR

Molecular typing of *Mycobacterium tuberculosis* complex Isolated from pulmonary tuberculosis patients in central Ethiopia

4.1 Abstract

Introduction: Identification of the types of strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) complex causing tuberculosis (TB) could contribute to TB control program of specific geographic region as well as it could add knowledge onto the existing literature on TB worldwide.

Objective: The aim of the present study was to identify the species and strains of *M. tuberculosis* complex causing pulmonary tuberculosis in central Ethiopia using RD 9 based PCR reaction spoligotyping and RD9 24 loci mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR).

Methodology: A total of 281 isolates of *M. tuberculosis* complex were isolated from pulmonary TB cases visiting health institutions found in the districts located north and south of the Addis Ababa city. Isolation was done on LJ medium while identification of the species and strain was made using RD 9 based PCR reaction and spoligotyping. A total of 29 isolates of selected spoligotypes were characterized by 24-loci MIRU-VNTR.

Result: Of the total isolates 98.6% of the isolates were identified to be *M. tuberculosis* while the remaining 1.4% was identified as *M. africanum*. Further, typing lead to the identification of 90 different strains of *M. tuberculosis*. Of these strains, 32 were clustered consisting of 79.3% (223/281) isolates while the remaining 20.6% (58/281) strains were single (unique) consisting of only one isolate. Forty-five of the strains were registered in the SITVIT2 or SpolDB4 database in while the remaining 45 were not found in the database and hence were orphan strains. The dominant strains were SIT53, SIT149, and SIT54. Classification based on lineage showed that 86.8%, the isolates belonged to the Euro-American lineage.

The result of MIRU-VNTR showed that isolates with similar spolygotype pattern showed difference in their copies of 24 MIRU loci. Accordingly, there were

polymorphisms in the number of copies of the 24 loci of the 4 isolates belonged to SIT53, the 6 isolates belonged to SIT 149,the 3 isolates belonged to SIT 37 and the 2 isolates belonged to SIT 54. Majority of the isolates belonged to Euro-American lineage. The MIRU loci 10, 24, 31, Mtub 39 and QUB 26 were the most discriminatory. The MIRU dendrogram grouped 29 of the isolates into 5 clonal groups; two of the groups had a single isolate each, the 3rd Group had 4isolates while the 4th and 5th groups had 8 and 15, isolates respectively.

Conclusion: The majority of the isolates were clustered strains, which could suggest the possibility of on-going active transmission in the population of the study areas. Strains that had the same spoligotype patterns had different MIRU-VNTR profiles upon typing using a 24 loci-based MIRU-VNTR typing revealing the better resolution power of MIRU-VNTR as compared to spoligotyping.

Keywords: Diversity of strain, *M. tuberculosis*, central Ethiopia.

4.2 Introduction

Tuberculosis (TB) is one of an infectious disease, affecting millions of people worldwide. According to the WHO TB Report in 2014, there were 9.6 million new TB cases and 1.5 million TB deaths annually (WHO, 2015). The spread of human immunodeficiency virus (HIV) and drug-resistant TB have exacerbated the situation.

Effective TB control program requires understanding of its epidemiology including the strains of *M. tuberculosis* complex (MTBC) circulating in the population (Van Soolingen, 1998). Molecular epidemiological studies have been used to provide valuable information on the spread of tubercle bacilli in outbreaks (Paranjothy *et al*., 2008) and also contributed to study the transmission dynamics of TB (Tostmann,

2008). Moreover, such studies can help in distinguishing exogenous reinfection from endogenous reactivation (Dobler *et al.,* 2008). Additionally, molecular epidemiological studies can be used to identify laboratory cross contamination (Allix *et al*., 2004) and to track the geographic distribution and spread of clones, including MDR strains (Mathema *et al.,* 2006). Furthermore, molecular typing has shown the large differences in pathobiological properties of MTBC species (Allix-Beguec *et al.,* 2010).

Molecular epidemiology of TB has been widely used to study and to trace TB transmission (van Soolingen *et al*., 2003). DNA fingerprinting of *M. tuberculosis* has gained increased acceptance as a useful tool for molecular epidemiological investigations (van Deutekom *et al*., 2004). Previously, a large number of DNA fingerprinting methods based on various genetic markers have been developed (Mazars *et al*., 2001; van Soolingen, 2001). Advances in molecular epidemiology like the development of 24-loci MIRU-VNTR typing provide a powerful tool to analyze MTBC population structure and transmission dynamics mycobacterial species locally and globally, which provides valuable information for the development of effective TB control strategies.

MIRUs are located mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome (Mazars *et al*., 2001; van Soolingen, 2001). The lengths of MIRU repeat units are in the range of 50-100 bp. MIRU- VNTR genotyping basically rely on PCR amplification using primers specific for the flanking regions of the VNTRs and on the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, these sizes reflect the numbers of the amplified VNTR copies. The final result is a numerical code, corresponding to

the repeat number in each VNTR locus. Such numerical genotypes are intrinsically portable and are thus particularly convenient for both intra- and inter-laboratory comparative studies (Mazars *et al*., 2001). The 24-loci MIRU-VNTR genotyping allows for a high-resolution discrimination of isolates for epidemiological studies and a valid phylogenetic strain classification (Supply *et al*., 2006).These loci display polymorphisms in MIRU copy number among nonrelated *M. tuberculosis* isolates. MIRU-VNTR typing has the advantages of being faster, and appropriate for virtually all *M. tuberculosis* isolates, including strains that have a few IS*6110* copies (Mazars *et al.*, 2001).

Tuberculosis (TB) is one of the most challenging diseases for developing countries mainly in Sub-Saharan Africa including Ethiopian. The World Health Organization global reports on TB showed that Ethiopia is among the top high TB burden countries (WHO, 2015). However, little data is available about the strain diversity of *M. tuberculosis* in most of these countries. Identification the types of strains of *causing* TB in a specific geographic region could contribute to the strengthening the TB control program. In addition, it could help in adding additional knowledge on the existing literature on TB worldwide. Therefore, the objective of the present study was to identify the species and strains of MTBC causing pulmonary TB and to assess the genetic diversity and phylogenetic analysis of selected *M. tuberculosis* isolates from central Ethiopia.

4.3 Result

4.3.1 Demographic Characteristics of the Study participants

Among 338 smear positive sputum samples, 297 (87.9%) samples were confirmed as culture positive pulmonary TB, 41 (12.1%) were culture negative or contaminated and thus excluded from further analysis. A total of 297 *M. tuberculosis* isolates were utilized to carry out RD9-PCR and spoligotyping analysis of which 281 gave valid spoligotyping data while the remaining 16 isolates did not give any pattern up on spoligotyping. One hundred thirty (46.2 %) of 281 isolates obtained from female while 151 (53.8 %) were isolated from males. Classification of the study participated on the basis of age showed that 99 (35.2%) were between 18 and 28 years of age, majority (47.7%; 134/281) were originated from Woliso and its surroundings (table 4.1).

Table 4.1: Background characteristics tuberculosis patients from whom the *M. tuberculosis* isolates were obtained.

Background characteristics	**Number (%) of isolates**
Sex	
Male	151(53.7)
Female	130(46.3)
Age, Years	
18-28	99(35.2)
29-39	72(25.8)
40-50	74(26.3)
>50	36(12.8)
History of anti-tuberculosis treatment	
Previously treated	10(3.6)
Not previously treated	271(96.4)
Region	
Woliso	134(47.7)
Fiche	97(34.5)
Atat	50(17.8)

4.3.2 Region of difference (RD) 9-based polymerase chain reaction (PCR)

The isolates were analyzed using RD9 PCR and the result indicated that majority (98%) of the isolates had intact RD9 implying that the isolates were *M. tuberculosis* (Figure 4.1).

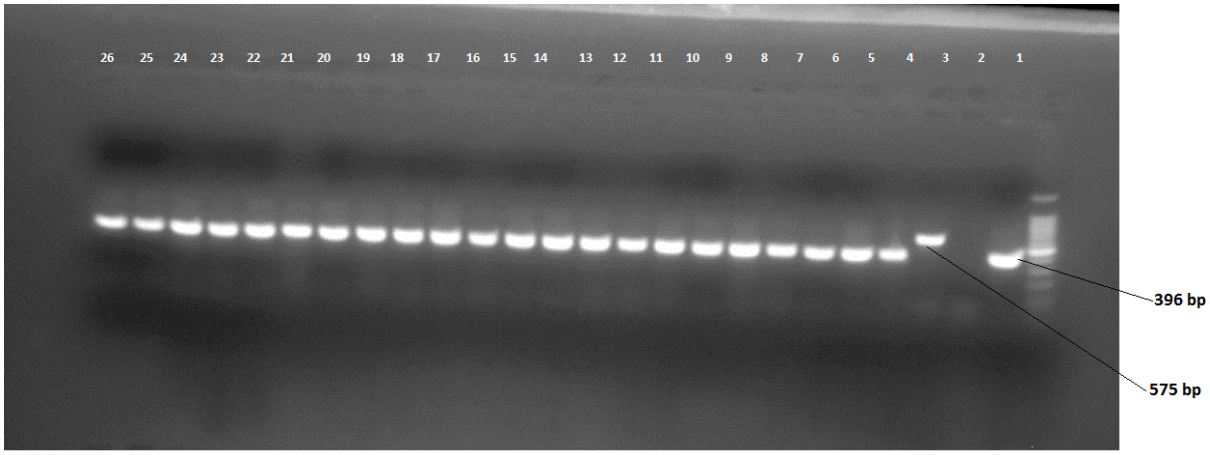

Figure 4.1: Picture of gel electrophoresis for RD9 deletion typing of *M. tuberculosis* isolates. Lane 1, is a ladder; Lane 2, is *M. tuberculosis* control; Lane 3, is Negative control (molecular grade H_2O); Lane 4, is *M. bovis* control ; lane 5-26, is culture isolate of *M. tuberculosis* collected from the study area.

4.3.3 Spoligotyping result

Spoligotyping of 281 isolates yielded 90 different spoligotype patterns. The isolates were grouped into 32 clustered strains containing of 223 (79.3%) isolates and 58 (20.6%) single (unique) strains (Table 4.2 and Table 4.4). The overall diversity of the isolates was 32.4%. Out of the 90 spoligtype pattern, 45 were registered in the

international data base (Table 2) and the remaining 45 were not found in the database (Table 4.3). The dominantly identified strains were SIT 53, SIT 149, and SIT 54 consisting of 43 isolates, 37 isolates, 34 isolates, respectively (Table 4.2). Classification of the spoligotype patterns using TB-insight RUN TB-Lineage showed that 86.8%, 6.4%, 5.3%, 1.4% of the isolates belonged to the Euro-American lineage, East-African-Indian, Indo-oceanic and *M. africanum,* respectively (Table 4.2).

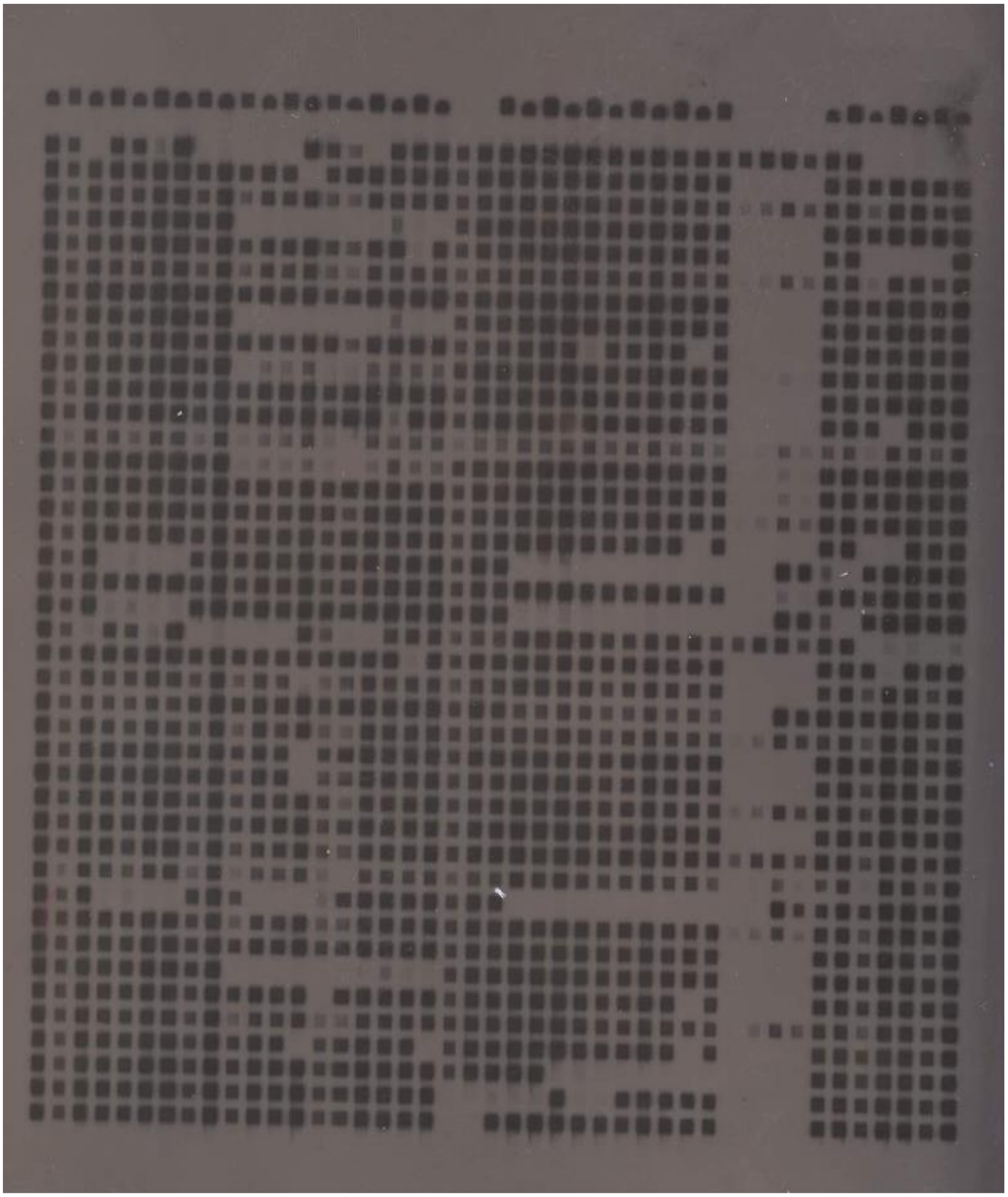

Figure 4.2 Representative spacers patterns obtained by the spoligotyping. The presence and absence of spacers was visualized on the film as black and white squares, respectively.

Lane 1 and 43 *M. tuberculosis* H37Rv as a positive control; Lane 2 water as a negative control; Lane 3 and 23 *M. bovis* as a positive control; the remaining others were the collected isolates

Table 4.2: Description of 45 shared-types (SITs;n=224 isolates) which have already been registered in the SITVIT2 or SpolDB4 database and corresponding spoligotyping defined lineages/sub-lineages starting from a total of 281 *M. tuberculosis* strains isolated in central Ethiopia.

SIT	Isolates with similar pattern	CBN* Lineage	SITVIT2 Lineage /sublineage	Octal number	Binary format
54	34	EA	Manu2	777777777763771	
53	43	EA	T1	777777777760771	
149	37	EA	T3-ETH	77700377760771	
37	15	EA	T3	777737777760771	
523	7	IO	Manu1	777777777777771	
817	4	EA	H3-Ural-1	777777777420731	
1690	4	EA	Manu2	777777777762771	
1821	9	EA	T	777347777760771	
2731	5	EA	Manu2	777777777767771	
52	2	EA	T2	777777777760731	
25	7	EAI	CAS1-Delhi	703777740003171	
26	2	EAI	CAS1-Delhi	703777740003771	
121	4	EA	H3	777777775720771	

137	2	EA	X2	777776777760601	
289	2	EAI	CAS1-Delhi	703777740003571	
47	2	EA	H1	777777774020771	
777	2	EA	H3-Ural-1	777777777420771	
336	4	EA	X1	777776777760731	
41	5	EA	Turkey	777777404760771	
119	2	EA	X1	777776777760771	
1688	2	EA	LAM	777777403760771	
50	2	EA	H3	777777777720771	
1634	1	EA	Manu2	777777777723771	
602	1	EA	H1	777777770000771	
910	1	M-africanum	AFRI	700000007177771	
1590	1	EAI	CAS1-Delhi	703777340003771	
357	1	EAI	CAS1-Delhi	703777740000771	
1197	1	EAI	CAS1-Delhi	703777740203171	
21	1	EAI	CAS1-Kili	703377400001771	
134	1	EA	H3	777777777720631	
584	1	EA	T2	777775777760731	

764	1	EA	H3	777757777720771	
4	1	EA	T1-RUS2	000000007760771	
157	1	EA	T3	777737777760471	
462	1	EA	T	777777777560771	
73	1	EA	T2	777737777760731	
504	1	EA	T3	777737737760771	
393	1	EA	T	777757777760771	
281	1	EA	T	777775777760771	
699	1	EA	H3	677777777720571	
102	1	EA	T	777703777760771	
966	1	EA	T	775777777760771	
93	1	EA	LAM5	777737607760771	
3134	4	EA	H3	777737377720771	
3900	3	EA	Manu2	777347777763771	

A total of 281 isolates grouped into 90 different spoligotype patterns. Of the 90 spoligtype pattern, 45 were registered in the international database while 45 of the strains were not found in the database. Of these 90 strains, 32 were clustered strains consisting of 223 (79.3%) isolates

while 58 strains were found as single isolate. The dominantly found strains were SIT 53, SIT 149, and SIT 54 consisting of 43, 37 and 34 isolates, respectively. Classification of the spoligotype patterns using TB-insight RUN TB-Lineage showed that 86.8%, 6.4%, 5.3%, 1.4% of the isolates belonged to the Euro-American lineage, East-African-Indian, Indo-oceanic and *M. africanum*, respectively.

Key: EA=Euro-American; EAI=East-African Indian; IO=Indo-Oceanic; MA= *M. africanum*; MB= *M. bovis*; CBN=conformal Bayesian network; KBBN =knowledge based Bayesian network.

Table 4.3: Description of 45 orphan strains (N=57) and corresponding spoligotyping defined lineages/sub-lineages recorded among *M. tuberculosis* strains starting from a total of 281 *M. tuberculosis* isolated in central Ethiopia.

SIT	Isolates with similar Pattern	CBN Lineage	SITVIT2 Lineage/ Sub-lineage	Octal number	Binary format
Orphan	3	IO	Manu1	777777777757771	
Orphan	2	EA	T3	006737777760771	
Orphan	2	EA	T3	006737737760771	
Orphan	2	EA	Manu2	777777777743771	
Orphan	2	EA	T3	776737677760771	
Orphan	2	EA	T3	776737777760771	
Orphan	2	EA	Manu2	777747777763771	
Orphan	1	IO	EAI7-BGD2	777777770003671	
Orphan	1	IO	EAI7-BGD2	777777770003471	

Orphan	1	EA	H1	777777760420731	
Orphan	1	EA	H4-Ural-2	501777577420431	
Orphan	1	EAI	CAS1-Delhi	703417740003771	
Orphan	1	EAI	CAS1-Delhi	703437740003771	
Orrphan	1	EAI	CAS1-Kili	701377404000771	
Orphan	1	EA	T3-ETH	777040377760771	
Orphan	1	EA	Turkey	777617404760771	
Orrphan	1	EA	Turkey	777377404240771	
Orphan	1	M-A	Manu2	575347777763661	
Orphan	1	M-A	Manu2	775347777763671	
Orphan	1	EA	Manu2	777777777763671	
Orphan	1	EA	Manu2	575347577762671	
Orphan	5	EA	Manu2	777757777763771	
Orphan	1	EA	Manu2	577347777762671	
Orphan	1	EA	Manu2	777777777763760	

Orphan	1	EA	Manu2	777747777762771	
Orphan	1	IO	Manu2	757777774243471	
Orphan	1	EA	Manu3	777347777770371	
Orphan	1	M africanu	Manu1	775347577777771	
Orphan	1	IO	H1	555347430000261	
Orphan	1	EA	T	777345777760671	
Orphan	1	EA	T	777347777777771	
Orphan	1	EA	T	777347777760671	
Orphan	1	EA	T	777245777760771	
Orphan	1	EA	T	776737437760771	
Orphan	1	EA	H3-Ural-1	575347777740671	
Orphan	1	EA	H3-Ural-1	773777776000771	
Orphan	1	EA	T2	701767777420531	
Orphan	1	EA	T	777755777760731	
Orphan	1	EA	T	577347777740671	

Orphan	1	EA	T	577347777760771	
Orphan	1	EA	T	577477777760771	
Orphan	1	EA	T	777047777760771	
Orphan	1	EA	T	557347437740661	
Orphan	1	EA	T1-RUS2	760002777760771	
Orphan	1	EAI	Manu2	777747777763671	

Of the 90 spoligtype pattern, 45 patters consisting of 57 isolates did not match with the patterns available in the database (orphans)

Key: CBN= conformal Bayesian network; EA=Euro-American; IO= Indo-Oceanic; EAI= East African-Indian; MA= *M. africanum*

4.3.4 Distribution of Strains and Lineages in the Study Area

Of the 281 isolates typed, 134(47.7) were originated from Woliso and its surroundings, with clustering rate of 83.6% (112/134), 97(34.5) were originated from Fiche with clustering rate of 72.2% (70/97) and the remaining were from Atat town with clustering rate of 82%(41/50). This finding did not show statistically significant difference in the proportion of clustering across the three source sites of the isolates (p-value = 0.163). Similarly, there was not significant association of the source site of the isolate with major lineage identified by CBBN (p-value = 0.877) as well as the type of dominant isolate (p-value = 0.109). The proportions of occurrence of the dominant lineage (Euro-American) at Woliso, Fitche and Atat towns were 85.8% (115/134), 88.6% (86/97) and 86 % (43/50), respectively (Table4).

Table 4.4: Distribution of strains and clustering rate in the Study Area (N=281).

Characteristics of the isolates	Number(%) of isolates in the study sites			P-value
	Weliso	Atat	Fiche	
Total spolygotyped isolates	134	50	97	
	(47.7)	(17.8)	(34.5)	
Clustered isolates versus single				
Clustered	112	41	70	
Single	22	9	27	0.163
Clustering rate	83.6%	72.2%	82%	
Major lineage by CBBN				
EA	115	43	86	
EAI	10	4	4	0.877
IO	7	2	6	
MA	2	1	1	
The three dominant Strains				
SIT 53	19	6	18	
SIT 149	17	9	11	
SIT 54	21	8	5	0.109
Orphan strains	21	10	26	

Key: EA=Euro-American; EAI=East-African Indian; IO=Indo-Oceanic; MA= *M. africanum*; CBN=conformal Bayesian network.

4.3.5 Genotyping of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit–variable number tandem repeat

The result of this study indicated that isolates with a similar spolygotype pattern had different profiles of MIRU-VNTR when typed with the MIRU-VNTR. Accordingly, there were polymorphisms in the number of copies of the 24 loci of the 4 isolates belonged to SIT 53, the 6 isolates belonged to SIT 149, the 3 isolates belonged to SIT 37 and the 2 isolates belonged to SIT 54.

According to this MIRU result all of the 29 isolates were distinct in their MIRU loci profile and there was no formation of genetic groups with 100% similarity. The MIRU loci 10, 24, 31, Mtub 39 and QUB 26 were the most discriminatory (Table 4.5 and Figure 4.3). Loci Mtub 39 was the most polymorphic with eleven different copies, followed by loci MIRU 31 with 9 different copies. MIRU 24 and QUB26 consists eight different copies each. MIRU loci 10 consists 7 different copies. The MIRU Loci 04 and MIRU loci 23 showed similarity in 96.6% (28/29) isolates, and Mtub30 and Qub-4156 showed similarity in 93% (27/29) isolates (Table 4.5 and Figure 4.3).

Table 4.5 Comparison of spolygotyping and MIRU

No of isolates	SIT	CBN Lineage (Label) Using spolygotyping	Copy number at 24 MIRU Locus	CBN* Lineage Using MIRU
4	53	EA (T1)	224121126342143334253624	EA
			25a681931a38772636249324	EA
			222221833346143332253521	EA
			223161633a24242342253426	EA
6	149	EA (T3-ETH)	224121133312143345253622	EA
			424141133412143344253a26	EA
			224121133312143345253622	EA
			225221533323322432254612	EA
			124121133312143344253d21	EA
			222221334641143b32252424	IO
3	37	EA (T3)	222221433j41243332253424	EA
			222221533341243332253424	EA
			222221313241142332253426	EA
2	54	EA (Manu 1	224121133312143325253429	EA
	54		224121136342143374253026	EA
1	1690	EA (Manu2)	224c21533333133346252f2b	EA
1	777	EA (H3- Ural-1)	220121133322143344253122	EA
1	121	EA (H3)	224211523343233344253622	EA

1	3134	EA (H3)	224211533463232324234621	EA
1	289	EAI (CASI-Delhi)	225221483323224342254d2c	EA
1	2731	EA (Manu2)	224121333312143344253629	EA
1	137	EA (X2)	224221533332142344253522	EA
1	Orphan	IO (EAI7-BGD2)	226121133322244444252722	EA
1	Orphan	EA (Turkey)	225221433323224322254621	EA
1	Orphan	EA(T3)	226a21633341642332258424	IO
1	Orphan	EAI (CASI-Delhi)	224221733k23424442254832	EA
1	Orphan	EA (T1)	22ae2133324124333225b421	EA
1	Orphan	EA (T3)	224211533343233344253622	EA
1	Orphan	EA-(T3)	622265363i31246c62693693	IO

The MIRU profile was based on sequence recommended by CDC in TB/ insight and consisted of MIRU 02, 04, 10, 16, 20, 23, 24, 26 ,27,31,39,40, Mtub 04,ETRC,Mtub21,QUB_11b, ETRA, Mtub 29,Mtub 30, ETRB, Mtub 34, Mtub 39,QUB 4156,and QUB 26.The letters are a:11; b:12; c;13; d:14; f:15; g:16; h:17; i:18; j;19.

Key: EA=Euro-American; IO= Indo-Oceanic; EAI=East African-Indian; MA=*M. africanum.*; CBN= conformal Bayesian network

The MIRU dendrogram constructed using tool at http://www.miruvntrplus.org/MIRU/index is presented in Figure 4.3.The dendrogram grouped the 29 isolates into 5 clonal groups (Figure 4.3 and figure 4. 4) two of the groups had a single isolate each, the 3rd Group had 4 isolates while the 4th and 5th groups had 8 and 15 isolates, respectively

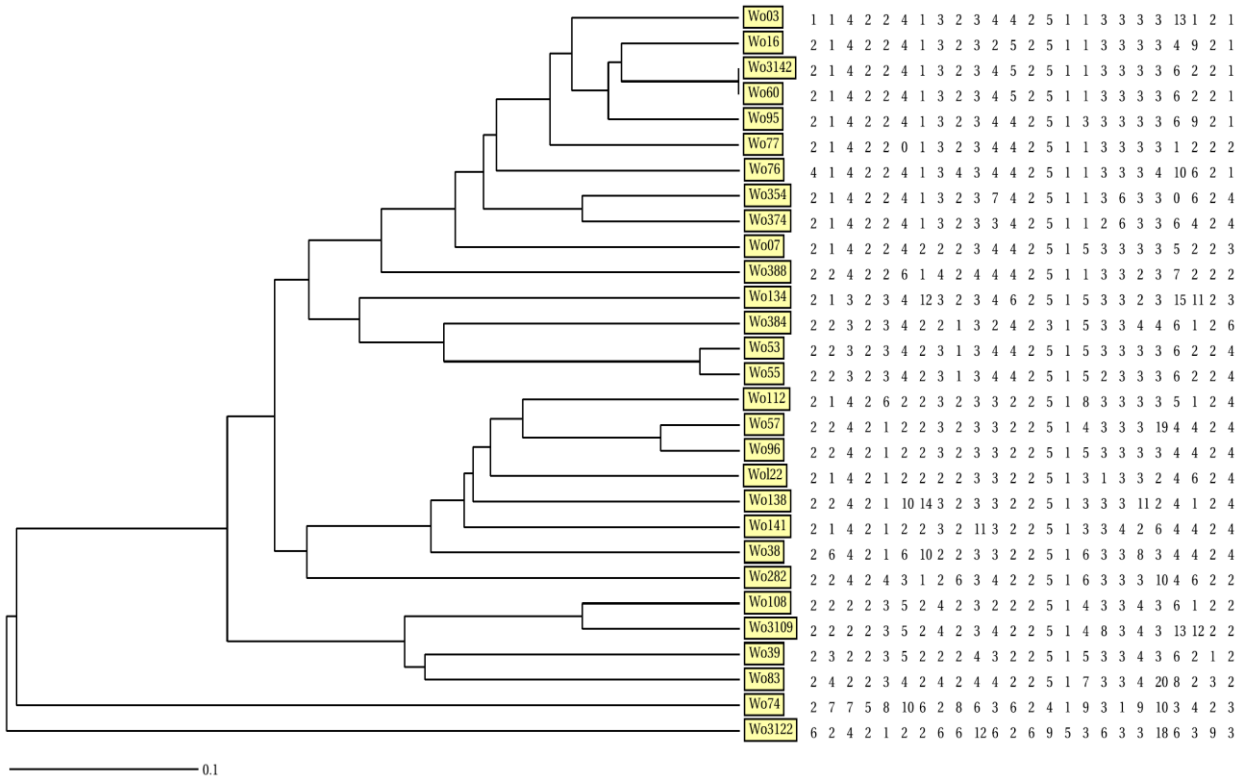

Figure 4.3 Dendrogram produced with the results obtained by MIRU profile (The 24 MIRU loci procedure was according to web tool at http://www.miru vntrplus.org/MIRU/found in the appendix). Five clonal groups were observed in the figure. Two groups consisted of a single isolates each observed far from other groups. The 3rd group consisted of 4 isolates, while the 4th and 5th groups consisted of 8 and 15 isolates respectively.

According to UPGMA tree the 29 isolates grouped into 5 clonal groups (Figure 4.4). Two groups consisted of a single isolates each, which were closely related to smooth ancient strain known as *M. Canettii* (figure 4.4). The 3rd group consisted of 4 isolates, while the 4th and 5th groups consisted of 8 and 15 isolates respectively. Most of the tested isolates were closely related to strainH37Rv.

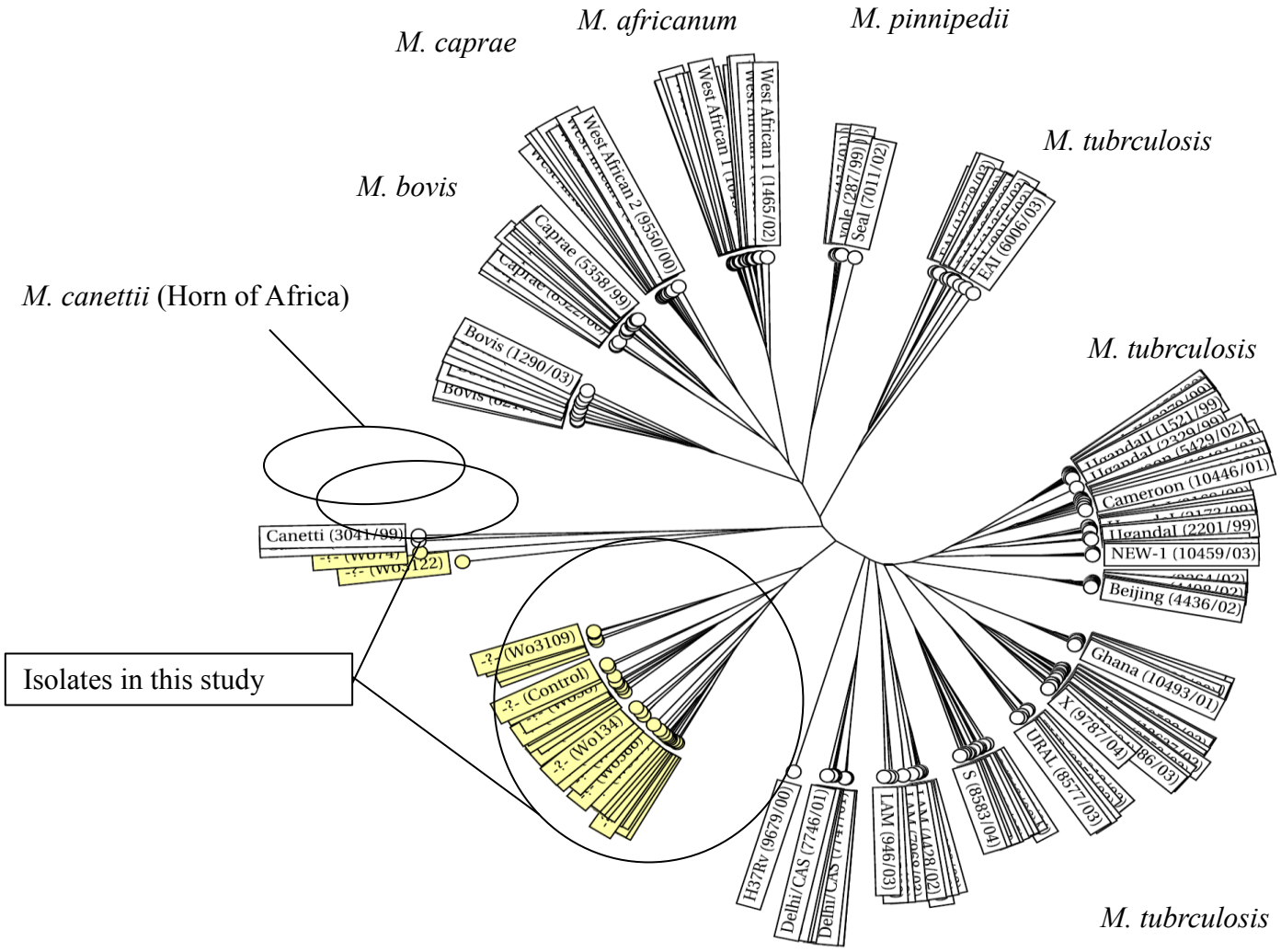

Figure 4.4. UPGMA tree based on the copy numbers of MIRU-VNTR 24-loci comparison with the reference database**.** Two groups consisted of a single isolates each, which were closely related to smooth ancient strain known as *M. Canettii*. The other groups were related to *M tuberculosis*

4.4. Discussion

In the present study, 281 pulmonary TB cases were recruited from three towns and their surroundings in central Ethiopia for the isolation and identification of the species and strains of MTBC causing pulmonary TB in the central Ethiopia. The isolation of was made from the sputum of patients and cultured on LJ medium while RD9-based PCR and spoligotyping used for identification of the isolates at the species and strain levels, respectively. An online tool Run TB-Lineage http://tbinsight.cs.rpi.edu/run_tb_lineage.html was also used for grouping lineages using a conformal Bayesian network (CBN) analysis and sub lineage using knowledge based Bayesian network (KBBN).

A total of 90 different types of spoligotype patterns were identified. Out of these 90 patterns, 45 patterns, consisting majority of the isolates, matched with the patterns registered in the SITVIT2 database while the remaining 45 patterns did not match with the patterns registered in the SITVIT2 or SpolDB4 database. Similar studies that have been conducted in Ethiopia, and thus all the geographic regions of the country have not been covered and as a result all the circulating strains of *M. tuberculosis* have not yet been registered to the SITVIT2 database. Over 75% of the isolates were clustered into strains with varies sizes of clustering while about a quarter of the isolates were found as single (Unique) strains. There are various assumptions with regard to such findings. Although spoligotyping has less discriminatory power in classifying strains, the finding of many isolates clustering in the same pattern could suggest the presence of an on-going transmission of *M. tuberculosis* infection in the specific geographic region. On the other hand, the isolation of many unique strains could suggest the introduction of new strains into that specific region and these strains did not spread in that specific geographic region.

The dominantly identified strains were SIT 53, SIT 149 and SIT 54 in order of decreasing frequency. Similar to this study, SIT54 was dominantly isolated by earlier studies conducted in the Addis Ababa City (Diriba *et al.,* 2013), central Ethiopia (Garedew *et al*., 2013) and in eastern central Ethiopia (Mulugeta *et al*., 2014). This strain has been mainly reported to the SITVIT2 and SpolDB4 database from South and East Asia, Middle East including Egypt and USA (SITVIT1 and Brudy *et al*., 2006). Another interesting finding in the study was, similar to other studies conducted in Ethiopia earlier (Diriba *et al.,* 2013; Mulugeta *et al*., 2014) the ancestor strain SIT 523 was found consisting of good numbers of isolates. SIT 523 is characterized by the presence of all 43 spacers and is the ancestor strain of *M. tuberculosis*. It is less likely that strains keep all the 43 spacers intact for a long duration of time since they have to adapt to different pressures through changing their genetic makeup, and hence it is likely that the presence of 43 spacers intact could also be due to mixed infection, which needs further investigation using more powerful molecular techniques.

The *M. tuberculosis* isolated by the present study were belonged to four major lineages including the Euro-American, East-African-Indian, Indo-oceanic and the *M. africanum*. The dominant Lineage was Euro-American Lineage consisting 86.8% of the isolates. This finding is in agreement with the earlier studies (Diriba *et al*., 2013; Garedew *et al.,* 2013 and Debebe *et al.,* 2014) conducted in different regions of Ethiopia. The 2nd and 3rd lineages under which the isolates grouped were East-African-Indian and Indo-oceanic lineage, respectively. Four isolates belonged to *M. africanum* Lineage. *M. africanum* has been reported to be an important cause of human TB in the west African countries including Guinea- Bissau (Källenius *et al.,* 1999), the Gambia (de Jong *et al*.,2006), Sierra Leone (Homolka *et al.,* 2008), Senegal (Viana-Niero *et al*.,

2001), Burkina Faso (Ledru *et al.*, 1996), Cameroon (Niobe-Eyangoh *et al.*, 2003), Nigeria (Cadmus *et al.*, 2006), and CôteD'Ivoire (Niemann *et al.*, 2004).

In the present study, the genetic diversity and phylogenetic classification of 29 *M. tuberculosis* Isolates were detected using MIRU-VNTR technique. An online toolhttp://tbinsight.cs.rpi.edu/run_tb_lineage.html was also used for grouping lineages using a conformal Bayesian network (CBN). In addition, http://www.miru-vntrplus.org/MIRU/was used to construct MIRU dendrogram and to classify the *M. tuberculosis* lineages.

In this study, 29 distinct genotypes were generated i.e. each of the 29 isolates were genetically different. Thus, none of the 29 isolates had 100% similarity. As observed in this study, isolates with similar SIT spoligotype pattern had distinct number of copies at their 24 MIRU Loci. For example, four isolates that belonged to SIT53 by spoligotyping had different MIRU-VNTR loci and thus found to be different genetically. Similar observations were made for the isolates that classified under the other SITs including SIT149, SIT37 and SIT54. This shows the greater discriminatory power of MIRU-VNTR in differentiating the *M. tuberculosis* isolates even those isolates with the same SITs. Comparable result was observed in another study performed in northern Ethiopia (Tesema *et al.*, 2013). This study showed that even the smaller number of *M. tuberculosis* isolates can present high genetic diversity. Majority of the isolates were belonged to major lineage Euro-American lineage (EA). Similarly, EA lineage was the dominant lineage by spolygotyping. Similar to this result, other study also showed that majority of the tested isolates were belonged to lineage four or Euro-American lineage, which was the most common lineage in Ethiopia.

Studies indicated that there is a hierarchy among the 24 polymorphic MIRU loci and it has been shown that loci 10, 23, 26, 31 and 40 generate the highest allelic diversity (Sola *et al*.,2001).In this study, MIRU loci 24, 31, Mtub 39,QUB 26 and MIRU 10 were highly polymorphic. None of the isolates had 100% similarity in their MIRU loci profiles although the MIRU loci 04 and MIRU loci 23 showed 96.6% similarity between the tested isolates. In addition, the MIRU loci Mtub30 and Qub-4156 showed 93% similarity between the tested isolates. The MIRU dendrogram grouped 29 of the isolates into five clonal groups. Two of the clonal groups consisted of one isolates each; the other two consisted of four and eight isolates while the remaining one consisted 15 isolates. Interestingly, two of the tested isolates were closely related to smooth ancient strain known as *M. Canettii*. *M. Canettii* is ancestor species and was isolated from TB patients in Djibouti or from individuals who spent some time at the horn of Africa (Koeck *et al*., 2010). The finding of *M. tuberculosis* closely related to *M. canettii* could suggest the presence of *M. canettii* in Ethiopia. However, it is not possible to make strong conclusion from this preliminary study as small sample size was used for MIRU-VNTR typing in the present study and this sample size is not representative as it undermines the internal and external validities of this study.

The limitation of this study is the use of only spoligotyping for typing of the majority isolates. In the study MIRU_VNTR was done only for 29 isolates. Since the discriminatory power of spoligotyping is low, the finding of this study could lead to an overestimation of clustering because of the failure to differentiate between mixed infections. Moreover, some of the strains claimed to be new in the present study may not be new and require identification by the use of typing techniques with a high discriminatory power like MIRU-VNTR.

CHAPTER FIVE

Drug sensitivity patterns of *Mycobacterium tuberculosis* isolates and associations between drug resistance phenotypes and genotypes of these isolates.

5.1 Abstract

Introduction Drug resistance tuberculosis (TB) and the emergence of multidrug resistant (MDR) isolates are significant concerns regarding TB control programs in several countries. **Objectives** This study was undertaken to evaluate the drug sensitivity pattern of *Mycobacterium tuberculosis* and to assess its association with strains and lineages of *M. tuberculosis.*

Methodology A total of 279 *M. tuberculosis* strains isolated from Central Ethiopia were tested for their drug sensitivity patterns to first line TB drugs using the conventional proportion method on Löwenstein-Jensen media. The association between drug sensitivity phenotype and strain type was assessed on 263 isolates of the 279 isolates.

Results Of the 268 *M. tuberculosis* isolates obtained from new cases, 78% (209) were susceptible to first line TB drugs, and 22.2% (59) bacterial isolates were resistant to at least one of the first line drugs. The highest mono-resistance (7.5%) pertained to streptomycin (STM). Remarkably, seven of eleven isolates (63.6%) previous treatment for TB were resistant to at least one of the first line drugs. The prevalence of MDR-TB was 1.5% (4/268) for newly identified TB cases, all of which were members of the Euro-American Lineage. There was no statistically significant association ($P > 0.05$) between drug sensitivity, and either strains, sub-lineages or main lineages of *M. tuberculosis*.

Conclusion A significant proportion of *M. tuberculosis* was resistant to at least one first line anti-TB drug. Moreover, the frequencies of resistance to either isoniazid or rifampicin were high compared to data that were previously reported in some part of the country.

Key words: Drug resistance, *M. tuberculosis*, proportional method, genotype of *M. tuberculosis*

5.2 Introduction

Tuberculosis (TB) causes illness in millions of people in each year and is ranked second to the human immunodeficiency virus (HIV/AIDS) as a leading cause of death from an infectious disease worldwide (WHO, 2014).

The emergence of drug-resistant TB poses a challenge to health care systems, especially in low income countries, and has exacerbated the situation as it pertains to the effective treatment of the disease worldwide. Inappropriate drug regimens, patient defaulting, previous anti-TB treatment, delays in the diagnosis and treatment of the disease, and primary infections with drug resistant or multi-drug resistant TB (MDR-TB) strains, poor infection control practices have been identified as major contributing factors for the spread of drug resistance TB (Andrews *et al.,* 2010).

Worldwide, an estimated 480,000 people developed MDR-TB. MDR-TB defined as resistant to two first line anti TB drugs, isoniazid and rifampicin. Globally, an estimated 3.5% of new cases and 20.0% of previously treated cases have MDR-TB (WHO, 2015). India, China and the Russian Federation account for more than 50% of the MDR-TB cases (WHO, 2013; WHO 2014; WHO, 2015). On average, an estimated 9.7% of patients with MDR-TB have extensively drug resistant TB (XDR-TB) (WHO, 2015). XDR-TB emerges when second-line drugs are misused to treat MDR-TB. According to the WHO report, XDR-TB had been reported in 105 countries (WHO, 2015). Fourteen of these countries reported more than 10% XDR-TB cases. Among those countries, the proportion of MDR-TB cases with XDR-TB was highest in Belarus (29% in 2014), Georgia (15% in 2014), Latvia (19% in 2014) and Lithuania (25% in 2013) (WHO, 2015).

Ethiopia is one of the 27 high MDR TB burden countries in the world (WHO, 2013; WHO, 2014; WHO, 2015). In 2014, the mortality due to TB was estimated to be 33 per 100,000 of the population (WHO, 2015). The same report showed that 1.6% of newly diagnosed TB patients and 12% of previously treated patients had MDR-TB. This data warrants for identification of drug resistant strains and monitoring their transmission in the community to contain their spread in the country. Specifically, it is of interest to know whether specific genotypes of *M. tuberculosis* (strains or lineages) are responsible for the development of the majority of drug resistances in a distinct geographical region. Therefore, the objective of the present study was to evaluate drug sensitivity patterns of *M. tuberculosis* strains isolated from central Ethiopia and to investigate if genotype of *M. tuberculosis* is associated with drug sensitivity.

5.3 Results

5.3.1 Socio-demographic characteristics

The socio-demographic characteristics of the study participants are summarized in Table 5.1. Out of the total of 279 isolates included in this study, 56.6% (155/279) were isolated from males while 44.4% (124/279) were isolated from females. The majority of the study participants (96.1%) were new patients. The mean age of the patients was 34.5 (Table 5.1).

Table 5.1. Socio-demographic characteristics of Central Ethiopian tuberculosis patients analyzed in this study.

Sex	Number (%)	95%Confidence Interval
Male	155 (55.6)	50.2-60.9
Female	124 (44.4)	39.1-49.8
Age group in year		
18-28	105 (37.6)	31.9-43
29-39	75 (26.9)	22.2-31.9
40-50	62 (22.2)	17.6-26.5
>50	37 (13.3)	9.3-17.6
Treatment history		
Previously treated cases	11 (3.9)	1.8-6.5
Newly diagnosed cases	268 (96.1)	93.5-98.2
Place		
Woliso	131 (47)	41.6-53.8
Atat	47 (16.8)	12.9-22.2
Fiche	101 (36.2)	30.1-42.3
Total	279 (100.0)	100.0-100.0

5.3.2 Drug resistance pattern to first line anti-TB drugs using the conventional method

The results of drug sensitivity tests for *M. tuberculosis* strains isolated from patients in Central Ethiopia are presented in Table 5.2. Drug sensitivity tests to four first line anti-TB drugs were performed for a total of 279 *M. tuberculosis* isolates. Out of the 268 *M. tuberculosis* isolated from newly diagnosed cases, 78.0% (209/268) were susceptible to the four drugs but 22.0% (59/268) of them were developed resistant to one or more drugs. Of the11 previously treated cases, 36.4% (4/11) were susceptible to all four drugs while 63.6% (7/11) were resistant to at least one drug. MDR was observed in 1.5% (4/268) of the new isolates. Mono-resistance was observed for 17.2% (46/268) of the new cases and 36.4% (4/11) of the cases previously treated for a TB infection. The highest proportion of mono-resistance in new cases was observed to STM (7.5%), followed by INH (6.3%), RIF (1.9%), and EMB (1.5%). The percentages for any resistance to RIF, INH, EMB and STM, including cases with resistance to more than one of the four drugs, were 3.6% (10/279), 10.4% (29/279), 3.5% (10/279) and 12.2% (34/279), respectively. Not a single strain was resistant to all four drugs tested (Table 5.2).

Table 5.2. Drug sensitivity patterns of *M. tuberculosis* isolated from central Ethiopia to first line anti-TB drugs using conventional method (n=279).

Drug resistance pattern	**New cases (n=268)**	**Treated cases (n=11)**
	n (%)	**n (%)**
All tested	268 (100)	11(100)
Susceptible	209(78.0)	4(36.4)
Any resistance	59(22.0)	7(63.6)
Mono-resistance	46(17.2)	4(36.4)
MDR	4(1.5)	0(0.0)
RIF only	5(1.9)	1(9.1)
INH only	17(6.3)	0(0.0)
EMB only	4 (1.5)	1(9.1)
STM only	20(7.5)	2(18.2)
RIF+INH	2(0.7)	0(0.0)
INH+STM	`4(1.5)	2(18.2)
INH+EMB	0(0.0)	1(9.1)
EMB+STM	3(1.1)	0(0.0)

INH+EMB+STM	1(0.4)	0(0.0)
INH+RIF+STM	2(0.7)	0(0.0)
Any RIF	9(3.4)	1(9.1))
Any INH	26(9.7)	3(27.3)
Any EMB	8 (3.0)	2(18.2)
Any STM	30(11.2)	4(36.4)

Key: MDR=multidrug resistant; RIF= rifampicin; INH= isoniazid; STM= streptomycin; EMB= ethambutol.

5.3.3 Association of drug resistance and demographic characteristics of the Study subjects

The result of the analysis of the association of the drug sensitivity patterns and socio-demographic characteristics of the subjects used as sources of the isolates is presented in Table 5.3. Anti-TB drug resistance was observed in male and female patients with 51.5% (34/66) and 48.5% (32/66), respectively and the difference in drug resistance between the two was statistically insignificance (P=0.450). High frequency of resistance was observed in the age group 18 to 28, significantly higher compared to other age groups (P= 0.005).

Table 5.3 Associations between demographic characteristics of the study subjects and drug sensitivity patterns of TB strains isolated from the subjects (n=279).

Characteristics	**Any drug resistance**		**COD (95% CI)**	**P- Value**
	Yes N (%)	**No N (%)**		
Sex				
Male	34(51.5)	121(56.8)	1	
Female	32 (48.5)	92(43.2)	1.238(0.712-2.153)	0.450
Age groups (in years)				
18-28	37(56)	68 (31.9)	0.350 (0.168- 0.761)	0.005
29-39	12(18.2)	63 (29.6)	0.353 (0.161-0.776)	0.010
40-50	10 (15.2)	52(24.4)	0.429 (0.172-1.071)	0.070
Above 50	7 (10.6)	30(14.1)	1	
Treatment history				
New patients	59 (89.4)	209(98.1)	1	
Previously treated	7(10.6)	4(1.9)	0.161(0.0460-0.570)	0.005
Study area				

Woliso	31(47)	100(47)	1	
Atat	8 (12.1)	39(18.3)	0.662(0.280-1.565)	0.348
Fiche	27(40.9)	74 (34.7)	1.177(0.648-2.138)	0.593
Total	**279**			

Key: COR= crude odds ratio; CI= confidence interval.

5.3.4 Relationships of drug-resistant phenotypes with *M. tuberculosis* genotypes

The relationship between the drug-resistant phenotypes and the *M. tuberculosis* lineages and strains was analyzed for 263 isolates and is summarized in Table 5. 4. The majority of the isolates were members of the sub-lineages T 49.8% (131/263) and Manu 27.0 % (71/263). The anti-TB drug resistances of the isolates in these two sub-lineages were 24.4% and 21.1%, respectively (Table 5.4). The majority (12/16) of the INH mono-resistant isolates and the majority (9/20) of the STM mono-resistant isolates were members of T sub-lineage.

Table 5.4. Drug resistance stratified by *Mycobacterium tuberculosis* lineage

Lineage	RIF Only	INH Only	EMB Only	STM Only	RIF+ INH	INH+ EMB	INH+ SM	EMB+ SM	INH +EMB +SM	INH+RIF +SM	Total resistance N (%)
CAS1-Delhi(14)		2			0	0					2 (14.3)
CAS1-Kili (2)		1			0	0					1 (50)
EAI- (2)		0		1	0	0					1(50)
H (26)	2	0	1	4	0	0		1			8 (30.8)
LAM5 (3)		0		1	0	0					1 (33.3)
Manu(71)	1	1	1	5	0	0	4	1		2	15 (21.1)
T(131)	2	12	2	9	2	1	2	1	1		32(24.4)
Turkey (6)		0		0	0	0					0
X(8)		0	1	0	0	0					1 (12.5)
Total	5	16	5	20	2	1	6	3	1	2	61

Key: RIF= rifampicin; INH= isoniazid; EMB=ethambutol ; STM= streptomycin.

The association between drug sensitivity patterns of *M. tuberculosis* isolates and the main lineages of *M. tuberculosis* is presented in Table 5.5. The frequencies of drug resistances were 19.8% (52/263), 1.1% (3/263), 1.9% (5/263) and 0.4% (1/263) in Euro-American, East African-Indian, Indo-Oceanic and *M. africanum*, respectively. Although the highest frequency of resistance was observed for the T sub-lineage, it was not statistically significant (P=0.404) compared to other sub-lineages. There was no significant association (P > 0.05) between drug resistance and either main lineage, sub-lineage or dominant strain.

Table 5.5. Association between drug resistance and the genotype of *M. tuberculosis* isolates (n=263).

Variable		Any drug resistance				
		Sensitive	**Resistant**	**Total**	**COD (95% CI)**	**P- value**
Major lineage by CBBN						
	EA	181	52	233	1	
	EAI	13	3	16	0.803(0.221-2.926)	0.740
	IO	7	5	12	2.486(0.758-8.159)	0.133
	MA	1	1	2	3.481 (0.214-56.609)	0.381
Sub-lineage/Clade						
	CAS1-Delhi	12	2	14	1	
	CAS1-Kili	1	1	2	6.000(0.257-140.045)	0.265
	EAI	1	1	2	6.000(0.257-140.045)	0.265
	H	18	8	26	2.667(0.481-14.789)	0.262
	LAM	2	1	3	3 .000(0.177-50.784)	0.447
	Manu	56	15	71	1.607(0.324-7.974)	0.562
	T	99	32	131	1.939(.4.12-9.129)	0.404
	Turkey	6	0	6		
	X	7	1	8	0.857(0.065-11.256)	0.907
Dominant strains						
	Orphan	41	12	53	1	
	SIT 53	34	9	43	0.904(0.341-2.401)	0.840
	SIT 149	25	12	37	1.640(0.639-4.204)	0.303
	SIT 54	27	4	31	0.506(0.148-1.734)	0.279
Clustering						
	No	39	18	57	1	
	Yes	163	43	206	0.572(0.298-1.097)	0.93

Key: EA=Euro-American; IO= Indo-Oceanic; EAI=East African-Indian; MA=*M. africanum*; CBN=conformal Bayesian network

5.4 Discussion

The present study was conducted to evaluate the drug sensitivity patterns of *M. tuberculosis* isolates. A total 279 *M. tuberculosis* isolates were included in this study. In addition, the association between drug sensitivity patterns and the genotype of *M. tuberculosis* was examined.

The result of this study revealed that 22% of the isolates from newly diagnosed TB cases and 63.6% of isolates from previously treated TB cases were resistant to at least one of the four anti-TB drugs investigated here. Comparable frequencies of resistance for newly diagnosed TB cases were reported from other areas in Ethiopia, such as Addis Ababa (Asmamaw *et al.,* 2008) and Eastern Ethiopia (Seyoum *et al.*, 2014); higher frequencies of resistance were reported from other regions (Mitike *et al* 1997; Agonafir *et al* 2010; Yimer *et al.*, 2012). Lower frequencies of drug resistances were reported from yet other Ethiopian regions (Gebeyehu *et al*., 2001; Abebe *et al*., 2012; Tessema *et al.,* 2012). Two studies from other East-African regions, the country Uganda (Sanchez-Padilla *et al*., 2013) and the city of Nairobi (Ndung'u *et al*., 2012), reported higher frequencies of resistance, 28.6% and 30%, respectively. The differences in overall prevalence of drug resistance among the different study settings could be due to difference in sample size, design of the study, study participant, access to health care facilities and effectiveness of TB control programs.

In the present study, 17.2% of the isolates from newly diagnosed cases and 36.4% from the re-treated cases were mono-resistant to any one of the four first line drugs, most commonly to streptomycin (7.5%) and isoniazid (6.3%). On the other hand, higher frequency (27.7%) of mono-resistance to any one of the four first line drugs was reported in another study conducted in the country (Getahun *et al.*, 2015). Isoniazid mono-resistance (6.3%) recorded by the present

study was comparable to that reported by studies conducted in other African countries such as the Central African Republic (Minime-Lingoupou *et al.,* 2011) and Somalia (Sindani *et al*., 2013), which reported 5.8% and 5.7% respectively. In an Eastern Ethiopian study (Seyoum *et al*., 2014), 9.5% mono-resistance to isoniazid was recorded. The isoniazid mono-resistance can increase the possibility of MDR-TB if rifampicin resistance also rises. Mono-resistance to isoniazid should be monitored in order to minimize the spread of MDR-TB strains.

In this study, the frequency of mono-resistance to rifampicin was 1.9% for newly diagnosed cases. This result is comparable to those of other studies conducted in Eastern Ethiopia (Seyoum *et al*., 2014) and Addis Ababa (Asmamaw *et al*., 2008). Even though rifampicin mono-resistance is relatively low according to the present study, monitoring appropriate use of this drug is also important to avoid the development of MDR-TB. Mono-resistance to ethambutol (1.5% of the cases) was higher compared to studies in Eastern Ethiopia (Seyoum *et al*., 2014), Addis Ababa (Agonafir *et al.,* 2010), Burkina Faso (Diandé *et al.,* 2009) and Uganda (Lukoye *et al*., 2011). In contrast, mono-resistance to ethambutol was higher in another study for Addis Ababa (3.5%) (Asmamaw *et al*., 2008).

The prevalence of MDR-TB in this study was 1.5%, in agreement with reports of other surveys conducted in Ethiopia (Gebeyehu *et al.,* 2001; Abebe *et al*., 2012). Other studies reported a higher frequency of MDR-TB (Agonafir *et al.,* 2010; Getahun *et al.,* 2015). Higher MDR cases observed in both studies may be due to small sample size and difference study participant because samples which were taken from TB specialized hospital (Agonafir *et al.,* 2010) might have higher MDR-TB than the finding of this current study. Control of MDR-TB requires an effective TB Control Program including a regular supply of anti-TB drugs, well-organized patient diagnosis, appropriate treatment, follow-up and good patient adherence. Higher

frequency of drug resistance reported in the age group 18-28 is in agreement with a WHO report (WHO, 2013). According to this WHO report two-thirds of TB cases are estimated to occur among young people. In Ethiopia, there are no regional bacterial culture and drug susceptibility testing facilities for routine diagnosis of drug resistance. Consequently, drug resistant TB is often diagnosed only after prolonged treatment with first-line anti-TB drugs and clinical recognition that treatment is failed. Therefore, identification of anti-TB drug resistant strains and understanding patterns of transmission of such strains in the community are important to control epidemiological outbreaks and further aggravation of drug resistance.

Studies showed that drug-resistant phenotypes are not equally distributed among *M. tuberculosis* genotypes (Anh *et al.,* 2000; Toungoussova *et al.,* 2002; Baker *et al.,* 2004 Hillemann *et al*. 2005). In this study, resistance to first-line anti-TB drugs was highest in Euro-American lineage and in T sub-lineage but the difference was not statistically significantly higher than the frequencies of resistance in other lineages and sub-lineages. Thus, in the present study, no association was observed between *M. tuberculosis* genotype and their resistance to the first line anti-TB drugs. Other studies performed in the north part of the country showed that Haarlem strains were more likely to be resistant to any of the four first-line anti-TB drugs compared to other lineages (Tessema *et al*., 2013).

Although the study has established the first data on drug resistance of *M. tuberculosis* circulating in specific sites of study area (North and Southwestern part of central Ethiopia), it has some limitations. One of the limitations was selection, the study not including all public health facilitates (in addition to hospitals) due to smaller patient flow in health centers. Besides, the study did not include the data of the possible association between HIV infection and anti-TB drug resistance.

CHAPTER SIX

Evaluation of the GenoType MTBDR*plus* Assay for Detection of Rifampicin and Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates

6.1 Abstract

Introduction: Multi-drug resistant tuberculosis (MDR-TB) is growing globally and becoming a major challenge for national TB control programs. Therefore, rapid identification of MDR strains of *Mycobacterium tuberculosis* and monitoring their transmission could contribute significantly to the control of TB.

Objective This study was carried out to evaluate the performance of the GenoType MTBDR*plus* assay for the detection of RIF and INH- resistant *M. tuberculosis* isolates.

Methodology: The conventional DST result of 279 *M. tuberculosis* isolates from the previous chapter were compared with data derived from the GenoType MTBDR*plus* assay.

Results: The sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of RIF-resistant *M. tuberculosis* isolates were 80.0% and 99.6%, respectively. Its sensitivity and specificity for the detection of INH-resistant *M. tuberculosis* isolates were 82.7% and 99.6%, respectively, whereas they were 75.0% and 100%, respectively, for the detection of MDR *M. tuberculosis* strains. The concordances of the GenoType MTBDR*plus* assay and the conventional DST for the detection of RIF and INH susceptibility were 80% (8/10) and 86.2% (25/29), respectively. Specific mutations were detected in 55.6% (5/9) of the RIF-resistant isolates, with the highest mutation rate (33.3 %) for *rpo*B gene (Codon S531L). For INH-resistant isolates, the highest mutation rate (88.8%) related to a *kat*G mutation (Codon S315T1).

Conclusion: GenoType MTBDR*plus* assay has high sensitivity and specificity for the detection of RIF and INH- resistance. These preliminary data supports the notion that the assay should be considered as an alternative to the DST for the characterization of MDR in *M. tuberculosis* isolates and the control of TB.

Keywords: Geno Type MTBDR*plus* assay; Drug resistance tuberculosis; Gene mutation.

6.2 Introduction

Tuberculosis (TB) is one of the major global health problems, with high prevalence in developing countries. A major concern is multi drug-resistant TB (MDR-TB), which is defined as the resistance to isoniazid (INH) and rifampicin (RIF), two therapeutic compounds for first-line TB treatment. The emergence of strains resistant to major anti-TB drugs has increased the need for identifying rapid and simple methods to detect such resistances and their molecular basis in the *Mycobacterium tuberculosis* genome. Using such tests promises to improve the physician's decision to appropriately treat the disease in patients on an individual basis and allows better monitoring of the emergence of MDR-TB strains in distinct geographical regions, ultimately contributing to the prevention of the spread of resistant strains. Drug-susceptibility testing by conventional methods using solid media such as Löwenstein Jensen is time consuming because *M. tuberculosis* grows slowly in culture requiring several weeks to identify the pathogen and test its drug-resistance profile. Even with more automated fluid culture methods, the former method takes an average of 14 days. Two additional weeks are required to obtain information about the strain's drug susceptibility (Ahmad *et al*., 2009). Molecular methods for drug-resistance testing based on the identification of mutations in genes associated with drug resistance, such as the GenoType MTBDR*plus* assay, offer an effective, alternative method to determine drug-resistance strains (Ioannidis *et al.,* 2011). The GenoType MTBDR*plus* assay is a molecular-line probe assay containing probes specific for the *M. tuberculosis* complex, wild type as well as probes for common RIF and INH-resistance-conferring mutations. The assays are based on reverse hybridization of amplicons immobilized on membranes. The GenoType MTBDR*plus* assay detects mutations in the *rpoB*, *katG,* and *inhA* genes and delivers results with a rapid turnaround time of 48 -72 hours. Nearly all RIF-resistant strains contain mutations in the

rpoB gene, which encodes the RNA polymerase subunit β (Hilleman *et al.,* 2007). Mutations in the *katG* gene and *inhA* genes are related to the high-level and low-level INH resistance, respectively (Hilleman *et al*., 2007). More than 95% of the RIF-resistant strains harbor a mutation within an 81-bp region of the *rpoB* gene from Codons 507 to 533 and this is region is named the RIF resistance-determining region (RRDR**) (**Telenti *et al.,* 1993; Kapur *et al.,* 1994). The highest level of RIF- resistance of the *rpoB* gene occurs in Codons 531 and 526. The *rpoB* gene mutations occur in Codons 511, 516, 518, 522, and 533 and cause low-level resistance to RIF-Resistance mutations are rarely identified in other regions of the *rpoB* gene (Kapur *et al.,* 1994**).**

Mutations causing INH-resistance are located in several genes. Several studies have demonstrated that 34.6-94.3% of INH resistance is most frequently associated with a mutation in the Codon 315 of the *M. tuberculosis* catalase peroxidase (*katG*) gene (Rattan *et al*., 1998; Hillemann *et al*., 2005). The *inhA* gene has 2.9% to 21.5% of its mutations in the promoter region (Kim *et al.,* 2003), and an additional 2% to 11.5% in the *ahpC-oxyR* intergenic region (Nikolayevsky *et al.,* 2004; Zhang *et al.,* 2005).

Ethiopia is one of the high MDR TB burden countries in the world. Annually, 2000-2500 MDR-TB cases are estimated to occur among all reported pulmonary TB cases. However, for example, in the year 2012, only 212 (10.1%) TB cases were detected (FMOH, 2014), indicating that the many of the expected MDR-TB cases remain undiagnosed and continue to spread in various communities. Therefore, improved monitoring of TB drug resistance with respect to the time required for detection of resistance and the sensitivity and specificity of detection of MDR-TB is important, and may benefit from molecular tests such as the GenoType MTBDR*plus* test. The

objective of this study was to evaluate the performance of the Genotype MTBDR*plus* test in detecting INH and RIF- resistant *M. tuberculosis* isolates in central Ethiopia.

6.3 Results

6.3.1 Sensitivity and specificity of the GenoType MTBDR*plus* Assay for Detection of Rifampin and Isoniazid -Resistant *Mycobacterium tuberculosis.*

Drug-sensitivity test was conducted on 279 *M. tuberculosis* isolates using the GenoType MTBDR*plus* assay and LJ medium-based proportion method. The result of the GenoType MTBDR*plus* assay showed that most isolates (96.8%) were susceptible to RIF; BY contrast susceptibility to INH (91.0%) was lower than that of RIF (Table 1). The GenoType MTBDR*plus* assay detected 9 (3.2%) of RIF-resistant, 25 (9.0%) INH resistant isolates. Moreover, 3(1.1%) isolates were found to be MDR by the Geno Type MTBDR*plus* assay.

Table 6.1: Drug Susceptibility Test result of Geno Type MTBDR*plus* assay (n=279).

New cases N=268 and treated cases N =11

Drug resistance pattern	New cases n (%)	Treated cases n (%)
RIF susceptible	260(97.0)	10(90.9)
INH susceptible	243(90.6)	11 (100)
Only RIF resistance	5(1.9)	1(10.0)
Only INH resistance	22(8.2)	0(0.0)
Any RIF resistance	8(3.0)	1 (10.0)
Any INH resistance	25 (9.3)	0(0.0)
MDR (RIF +INH) resistance	3(1.1)	0(0.0)

Key: RIF= rifampicin; INH= isoniazid; MDR-TB=multidrug resistance tuberculosis.

The results of the evaluation of the performance of the GenoType MTBDR*plus* assay in detecting drug resistance are summarized in Table 6.2. The sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of RIF-resistant *M tuberculosis* isolates were 80.0% and 99.6%, respectively. Its sensitivity and specificity for detecting INH-resistant *M. tuberculosis* isolates were 82.7% and 99.6%, respectively, whereas they were 75.0% and 100%, respectively, for detecting MDR *M. tuberculosis* strains. The concordances of the GenoType MTBDR*plus* assay and the conventional DST for the detection of RIF and INH susceptibility were 80% (8/10) and 82.7% (24/29), respectively. Furthermore, the concordance of the two tests in detecting MDR *M. tuberculosis* strains was 75%.

Table 6.2: Performance of GenoType MTBDR*plus* assay test LJ medium-based proportion method as a gold standard for the detection of resistance of *M. tuberculosis* to Rifampicin and Isoniazid

GenoType MTBDR *plus* assay		LJ DST result					
		Resistant	Susceptible	Sensitivity	Specificity	PPV	NPV
RIF	Resistant	8	1	80	99.6	88.9	99.2
	Susceptible	2	268				
INH	Resistant	24	1	82.7	99.6	96	98.4
	Susceptible	5	249				
MDR	Resistant	3	0	75	100	100	99.6
	Susceptible	1	275				

Key: DST= drug susceptibility testing; INH= isoniazid; RIF= rifampicin; MDR =multidrug resistance; PPV= positive predictive value; NPV =negative predictive value

6.3.2 Mutation patterns of RIF and INH produced by the GenoType MTBDR*plus* assay

Specific mutation was detected in five of the nine RIF-resistant isolates. Of these five, three isolates had mutation at Codon S531L whereas the remaining two isolates had mutation at Codon H526D. In the remaining four of the RIF-resistant isolates, WT 8 probe was missing with no gain in MUT 3 probes and considered as unknown.

In the GenoType MTBDR*plus*, INH resistance was detected using the probes of the *kat*G and *inh*A genes. Of the 25 INH-resistant isolates, *kat*G mutation occurred in 88.0% (22/25) of the isolates and in all of these isolates, specific mutations were observed at Codon S315T1 of the *kat*G gene. Mutations in the *inh*A gene occurred only in three INH-resistant isolates. Specific *inh*A mutations were observed in two of the INH-resistant isolates, which had mutation at Codon C15T whereas in the remaining one isolate the *inhA* WT 2 gene was missing without the presence of specific mutation band. These isolates are also considered unknown (Table 6.3).

Table:6.3 The mutation patterns of Geno Type MTBDR *plus* assay

***rpoB* mutations**	**Frequency**	***katG* mutations**	***inhA* mutations**	**Frequency**
S531L	3	S315T1	WT	22
H526Y	2	WT	C15T	2
UK (WT8 missed)	4	WT	UK (WT2 missed)	1
RIF resistant	9		INH resistance	25
RIF susceptible	270		INH susceptible	254

Key: UK = unknown mutation characterized by no hybridization to one or more wild type probes nor to any of mutation probes; WT =wild type; RIF =Rifampicin; INH =Isoniazid.

6.3.3 Band patterns of drug resistant *M. tuberculosis* strains

The genotypic profile of resistance to RIF and NIH was examined. Of the nine isolates that showed resistance to RIF by the GenoType MTBDR*plus*, the missing of WT5 was observed in two isolates, but missing of WT8 was observed in seven isolates. MUT2A was observed in two RIF-resistant isolates and MUT3 was observed in three RIF-resistant isolates. In the remaining four isolates, WT8 was missing without the corresponding mutation of MUT3.

Of the 25 INH-resistance isolates, 88% (22/25) isolates had a high-level resistance profile to the drug, which indicated absence of WT band WT1 and presence of corresponding mutation bands of MUT1 in the *katG* gene. The remaining three isolates showed a low-level resistance pattern; WT1 was missing in two isolates and WT2 was missing in one isolate of the *inh*A gene in INH-resistance, and there was a corresponding mutation in MUT1 in two isolates in the *inhA* regulatory region (Figure 6.1).

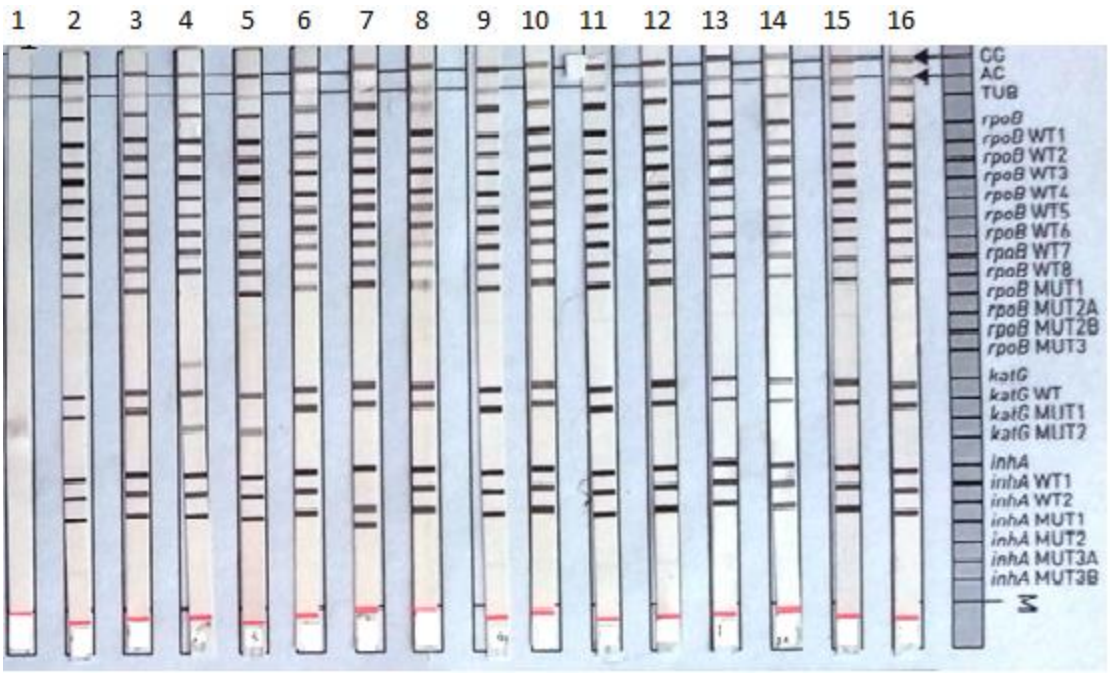

Figure 6.1. Representative DNA patterns obtained by the MTBDR*plus* assay. Lane 1, water as a negative control, lane 2,3,6,8,9,10,11,12,13,14,15 all are example of a pattern of RIF^s and INH^s, lane 4, example of a pattern of RIF^r and INH^r, lane 5, example of a pattern of RIF^s and INH^r with *kat*G mutation; lanes 7 example of a pattern of RIF^s and INH^r with *inh A* mutation; lane 16,H37Rv as a positive control.

Key: RIF^r =Rifampicin resistance; INH^r = isoniazid resistance; RIF^s = Rifampicin sensitive; INH^s = isoniazid sensitive.

The band patterns of isolates resistant to RIF and INH were observed in 6 of RIF mono-resistant, 22 of the INH mono-resistant and three MDR isolates. In the *rpo*B gene, the absence of WT5 was observed in two RIF mono-resistant isolates and there was a corresponding MUT2A in these two RIF mono-resistant isolates. The absence of WT8 was observed in four RIF mono-resistant isolates and in all of the three MDR isolates. The corresponding MUT3 was observed in

only on one RIF mono-resistant and in two MDR isolates. In the remaining four RIF-resistant isolates, WT 8 probe was missing with no gain in MUT 3 probes (Table 6.4).

In the case of INH-resistant strains, in the *kat*G gene, the missing WT1 was observed in 19/22(86.4%) of the INH mono-resistant isolates and in all of the three MDR isolates (Table 6. 4). The corresponding *kat*G MUT1 was observed in all of these 19 INH mono-resistant isolates and in all of the three MDR isolates. In the case of the *inh*A gene, the absence of WT1 was observed in only two isolates and there was a presence of the corresponding MUT1 in these two INH mono-resistant isolates. In the same *inhA* gene, WT2 was missing in one INH mono-resistant isolate without the presence of the corresponding mutation gene.

Table 6. 4: Band patterns of drug resistant ***Mycobacterium tuberculosis*** strains using Geno Type MTBDR*plus* assay

Gene	Band	Gene region/mutation	RIF mono resistance=6 N (%)	INH Mono resistance=22 N (%)	MDR=3
RpoB					
	WT1	506-509	6(100)	22(100)	3(100)
	WT2	510-513	6(100)	22(100)	3(100)
	WT3	513-517	6(100)	22(100)	3(100)
	WT4	516-519	6(100)	22(100)	3(100)
	WT5	518-522	4(66.7)	22(100)	3(100)
	WT6	521-525	6(100)	22(100)	3(100)
	WT7	526-529	6(100)	22(100)	3(100)
	WT8	530-533	2(33.3)	22(100)	0 (0)
	MUT1	D516V	0(0)	0 (0)	0(0)
	MUT2A	H526Y	2 (33.3)	0 (0)	0(0)
	MUT2B	H526D	0(0)	0 (0)	0(0)
	MUT3	S531L	1 (16.7)	0 (0)	2 (66.7)
KatG					
	WT1	315	6(100)	3 (14.5)	0(0)
	MUT1	S315T1	0 (0)	19(86.4)	3(100)
	MUT2	S315T2	0 (0)	0(0)	0(0)
inh A					
	WT1	-15/-16	6(100)	20(90.9)	3 (100)
	WT2	-8	6(100)	21(95.4)	3(100)
	MUT1	C15T	0 (0)	2 (9)	0 (0)
	MUT2	A16G	0 (0)	0(0)	0 (0)
	MUT3A	T8C	0 (0)	0(0)	0 (0)
	MUT3B	T8A	0 (0)	0(0)	0 (0)

WT =wild type; MUT = mutation.

6.4 Discussion

In this study, the performance of the GenoType MTBDR *plus* assay for the detection of RIF and or INH-resistant strains of *M. tuberculosis* was evaluated on 279 *M. tuberculosis* isolates, which were isolated from pulmonary TB patients at three towns and their surroundings in central Ethiopia. We observed that the GenoType MTBDR*plus* assay results had a good concordance with the conventional DST with additional advantage of a shorter turnaround time. The sensitivity and specificity of the GenoType MTBDR*plus* assay for detection RIF and or INH-resistant *M. tuberculosis* isolates were very good. The overall concordance of the GenoType MTBDR*plus* assay and the conventional DST method was 81.4%. The sensitivity and specificity of the GenoType MTBDR *plus* were 80.0% and 99.6%, respectively, for the detection of RIF resistance. Moreover, they were 82.7% and 99.6%, respectively, for the detection of INH resistance. The result of another study conducted in northwest Ethiopia reported sensitivity and specificity of 92.0% and 99.0%, respectively, for the detection of INH resistance whereas both sensitivity and specificity for the detection of RIF resistance were 100% (Tessema *et al*., 2012). A study in India reported sensitivity, specificity, positive predictive values and negative predictive value of 97.6%, 94.4%, 97.6%, and 94.4%, respectively, for detection of RIF-resistance but, the same study reported sensitivity and specificity values of 83.3% and 93.8%, respectively, for the detection of INH resistance (Singhal *et al*.,2012). Study in Uganda and France (Albert *et al*., 2010; Brossier *et al*., 2006) reported sensitivity of 100% for the detection of RIF-resistance.

In this study, the GenoType MTBDR*plus* assay identified RIF-resistance specific mutation by *rpoB* MUT probes which was be detected in five of the nine RIF-resistant isolates. Higher specific mutation on the *rpoB* gene was reported in another study in India (Singhal *et al.,* 2015) and in South African (Barnard *et al*., 2008).

The result of this study showed that of the five specific mutations of the *rpoB* gene, three had mutations at Codon S531L. The remaining 22.2% (2/9) had mutation at Codon H526D. A similar finding was reported by a study conducted in India (Raj *et al.,* 2013). In four of the nine RIF-resistant isolates, WT 8 probe was missing with no gain in MUT3 probes, which indicated the presence of a less common or rare mutation. Similarly, RIF-resistant isolates with the missing of WT 8 probe without any MUT band were reported in other studies from France, New Delhi and Vietnam (Brossier *et al*., 2006; Singhal *et al.,* 2012; Huyen *et al*., 2010). Other study conducted in northwest Ethiopia reported that all phenotypically defined RIF-resistant strains and MDR strains had mutations conferring resistance to RIF, and to INH i.e. MDR.

In this study, the frequency of mutation at Codon S531L occurred more in two of the three MDR strains and in one of the six RIF mono-resistant strains. However, higher frequency of mutation at Codon S531L was reported from South African and Indian in MDR and RIF mono-resistant strains (Barnard *et al.,* 2008; Singhal *et al.,* 2012).

In the Genotype MTBDR*plus* assay, resistance to INH is detected by probes of the *kat*G and *inh*A genes. The higher frequency of resistance to INH occurred due to mutation of the *KatG* gene whereas lower frequency of resistance is caused by the mutations in the promoter region of the *inhA* gene (Zhang and Yew, 2009). Of the 25 INH-resistant isolates, *kat*G mutation occurred in 88% (22/25) of the isolates. In all of these *22* isolates, specific mutations were found at Codon

S315T1 of the *katG* gene, which was also reported by other studies conducted in northwest Ethiopia (Tessema *et al.,* 2012) and in India (Singhal *et al.,* 2015). Some studies reported lower frequencies of mutation in *kat*G gene at Codon S315T1 from France, Uganda, and from South Africa (Brossier *et al*., 2006; Albert *et al.,* 2010; Barnard *et al.,* 2008).

Mutations in the *Inh*A gene occurred in only three of the 25 INH-resistant isolates, which is similar to the frequency of *inhA* mutation reported from the northwest part of Ethiopia (Tessema *et al*., 2012). Compared with this findings, a study from North India reported the occurrence of low frequency of INH-resistance mutation in the *Inh*A gene (Singhal *et al*., 2015). In contrast to these findings, higher *inhA* gene mutation was reported from Tunisia (Soudani *et al*., 2011) and Canada (Bolotin *et al*., 2009). Specific *inh*A mutations were found in two of the three INH-resistant isolates, which had mutation in Codon C15T, but in the remaining one isolate, the *inhA* WT2 gene was missing without the presence of a specific mutation band. In this study, five of the phenotypically defined INH-resistant strains had no mutations in the *katG* and *inhA* genes. This could suggest that there may be other mutations in other Codons of the *katG*, and *inhA* genes or presence of some unidentified mutations in other genomic regions such as *ahpC, kasA, furA*.

CHAPTER SEVEN

General Discussion, Conclusion and Recommendation

7.1. General Discussion

The present study conducted to characterize *M. tuberculosis* isolates at strain and a lineage (sub-lineage) level in central Ethiopia using spoligotyping and MIRU-VNTR. The dominant strains and lineages were identified from health institutions of three towns. Additionally drug sensitivity tests were conducted on *M. tuberculosis* isolates using the conventional proportional LJ and the Genotype MTBDR molecular methods. In connection, the performance of Genotype MTBDR test for the detection of RIF-resistant and INH resistant *M. tuberculosis* was evaluated by using the conventional proportional LJ as a gold standard.

Molecular typing of *Mycobacterium tuberculosis* complex

In this study, mycobacterial strains isolated from three different town of central Ethiopia were characterized using molecular tools. Although the covered study areas do not represent whole Ethiopia, about 300 isolates were typed and the strains identified could give insight into the population structure of *M. tuberculosis* in central Ethiopia.

The most prevalent *M. tuberculosis* strains were SIT53 (T1 clade), SIT 149 (T3-ETH clade) and SIT 54(manu2 clade) in decreasing order. Majority of the isolates were found in clustered strains similar to the earlier reports by other authors (Mihret *et al*., 2012; Diriba *et al.,*2013; Mulugeta *et al.,*2013), which could suggest recent transmission in the study area. Based on the SpolDB 4.0 database and SITVIT2 results, the most prevalent major lineage was the Euro-American Lineage. The findings of this study are in agreement with the results of other studies conducted in different regions of Ethiopia (Agonafir *et al.,*2010; Deribew *et al*.,2012; Mihiret *et al*.,2012; Garedew *et al.,*2013). Furthermore**,** similar to the results of this study, other studies in north

eastern and central Ethiopia (Garedew *et al.,* 2013; Mulugeta *et al.,* 2013) reported high frequencies of Manu clade. Investigators from Egypt reported a high frequency of Manu Clade (Helal *et al.,* 2009). SIT 54 (Manu2) has mainly been reported from South and East Asia, Egypt, and America. On the other hand, 45 spoligotype patterns could not match (were Orphans) with the patterns registered in the SITVIT2 or SpolDB4 database and thus these strains could be endemic to Ethiopia.

Genotyping of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit–variable number tandem repeat: MIRU-VNTR with 24-loci was applied to 29 randomly selected *M. tuberculosis* isolates. According to the MIRU-VNTR typing none of 29 had 100% similarity and isolates with the SIT had different MIRU-VNTR profiles. Thus variable levels of polymorphism were observed in the number of copies in their 24 loci between the isolates. This means that strains that had the same spoligotype (SIT) patterns had different MIRU-VNTR profiles upon typing using a 24 loci-based MIRU-VNTR revealing the better resolution power of MIRU-VNTR as compared spoligotyping.

Drug sensitivity patterns and its association with genotype

Prevalence of TB drug resistance is an indicator for the quality and performance of a national TB control program and offers an opportunity to assess the amount of resistant bacteria transmission in the community. Treating patients with MDR-TB requires expensive and toxic chemotherapy and often results in extremely low cure rates and increased mortality. Accurate detection of resistant strains is critical to provide appropriate treatment and to intercept the transmission of drug-resistant TB. This study demonstrated that 22% of patients with drug-resistant TB were due to recent transmission (new cases) while 63.6% of isolates from previously treated cases were

resistant to at least one of the four anti-TB drugs tested. In this study though RIF mono resistance seems low, it is important to manage the proper use of this drug because this drug is a surrogate marker of MDR-TB.

In this study majority of the drug resistant strains and all of the MDR isolates were isolated from patients with the age groups 18–28 years old. The high prevalence of drug resistance TB and MDR-TB in a young population group implies recent transmission of drug resistant strains which need a serious concern to control the transmission of drug resistant strains in the study area. In addition, from TB-control point of view, it is relevant to understand whether specific genotype families are overrepresented among drug-resistant cases and, in particular, if these resistant strains are successfully transmitted within the community. In this study, resistance to first-line anti-TB drugs was highest in EA lineage and in sub lineage T, Manu and Haarlem. All MDR were grouped in EA lineage and 3 of them were grouped in T- sub-lineage. Analysis and timely updates of the *M. tuberculosis* strain distributions in a given country and comparisons to worldwide patterns provides insights into transmission mechanisms, emergence of drug resistance and particularly virulent strains, and information relevant to the development of new diagnostics, drugs, and vaccines.

Evaluation of the GenoType MTBDR*plus* Assay for Detection of Rifampicin and Isoniazid Resistant *Mycobacterium tuberculosis*

The GenoType MTBDR*plus* provides comprehensive information about the mutations leading to resistance. The concordance of the phenotypic DST and GenoType MTBDR*plus* assay for the rapid detection of RIF-mono resistance, INH mono-resistance and MDR-TB resistance among isolates from PTB patients in the central Ethiopia. This study showed that high sensitivity and

specificity of GenoType MTBDR*plus* assay taking conventional LJ-based proportion method of DST as a gold standard. Therefore, it is preferable to use GenoType MTBDR*plus* assay as it is rapid and technically simple to perform.

The limitations of the GenoType MTBDR*plus* assay is it can identify only the most frequent mutations (some are not identified yet) and otherwise rely on the negative hybridization result with the WT-specific probe as a marker for additional mutations. Since not every mutation confers drug resistance, the significance of the mutation detected can be difficult to interpret. Moreover, the Genotype MTBDR*plus* test cannot identify those INH-resistant isolates that have resistance-conferring mutations other than in *katG and inhA*. Hence, the clinical utility of the test varies depending on the prevalence of the particular mutations at these loci.

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7.2. Conclusion and Recommendation

This study was conducted in three sites of central Ethiopia, which, is expected to have high TB cases because of higher population movement and over-crowding. In this study, pulmonary TB patients visiting health institutions in three areas of central Ethiopia and used as sources of sputum samples for the isolation of *M. tuberculosis,* and its identification at strain level. Clustered and unique stains of *M. tuberculosis* were identified. In addition, orphan strains were identified from the three districts. Clustering of strains could suggest to possibility of on-going active transmission while on the other hand the presence of unique stains shows the presence new strains which could be indigenous to Ethiopia and have not been known to the glob partly due to the absence of prior studies using molecular tools in the study areas.

In connection, strains that had the same spoligotype patterns had different MIRU-VNTR profiles upon typing using a 24 loci-based MIRU-VNTR typing revealing the better resolution power of MIRU-VNTR as compared spoligotyping.

In addition to identification of the strains of *M. tuberculosis*, drug sensitivity tests were conducted on these strains using the conventional and molecular methods. The result showed that a significant percentage of *M. tuberculosis* was resistant to at least one or more first line anti-TB drug. Moreover, resistance to either isoniazid or rifampicin was high as compared to frequencies of resistance reported earlier in other parts of the country. On the other hand, no association was observed between the genotype of *M. tuberculosis* isolates and their drug sensitivity patterns.

Lastly, the performance of Genotype MTBDR test was evaluated for rapid detection of drug resistance. Accordingly, GenoType MTBDR*plus* assay was observed to have high sensitivities

and specificities in detecting RIF and INH resistant as well as in detecting MDR *M. tuberculosis* isolates.

Based on the above conclusions the following points were recommended:-

1. Strengthening of the TB control strategies of the three areas (North Shewa and Southwest Shewa) of central Ethiopia so that the transmission of the disease is further minimized.
2. Further studies recommended using molecular methods with a better discriminatory power such as single nucleotide polymorphism (SNP) typing and MIRU-VNTRs, which allow splitting certain spoligotyping clusters in smaller sub clusters to differentiate new strains from mixed or polyclonal infections.
3. Creation of awareness in the communities and health professionals of the three districts on the proper use anti-TB drugs so as to minimize the occurrence drug-resistant strains
4. Further evaluation of GenoType MTBDR*plus* assay of the detection of drug resistance and promotion of its use in different health institutions.

Areas of further study

This study was conducted at three different sites in central Ethiopia and hence the its findings could not represent of the whole geographic area of central Ethiopia. Thus, in order to establish the realistic picture of the strain circulating in central Ethiopia a better representative study should be conducted

In this study, orphan strains, ancestral strains like *M africanum,* SIT523, *M Cannetti* were recorded. The presence of these strains in the study area requires further studies to generate additional findings molecular methods better resolution such as nucleotide polymorphism typing and mycobacterial interspersed repetitive units variable number of tandem repeats.

Limitation Of the study

1. The study did not include the data of the possible association between HIV infection and anti-TB drug resistance. Therefore, studies are required by including other possible risk factors for drug resistance such as HIV.

2. The study not evaluating the performance of the GeneXpert MTB/RIF test, because recently the assay is introducing into the country for routine TB laboratory diagnostic.
3. The third limitation of this study is that the drug sensitivity pattern of isolates is not done for second line drugs. Since drug resistance and multi-drug resistance isolates are found in the study, the presence of XDR-TB has to assessed.

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APPENDICES

Appendix I: Summary of the target primers.

A, Primers for Region of Difference 9

RD 9 FW	AACACGGTCACGTTGTCGTG
RD 9 INTREV	TTGCTTCCCCGGTTCGTCTG
RD 9 REV	CAAACCAGCAGCTGTCGTTG

B, Sploygotyping primers

DRa 5’-GGTTTTGGGTCTGACGAC-3’ (biotinylated5’ end) and

DRb 5’-CCGAGAGGGGACGGAAAC-3’

C, Primers for MIRU-VNTR

Table I Primers for MIRU-VNTR

Locus	MIRU	Repeat unit length, bp	Flanking size	PCR primers (5' to 3')
580	MIRU 04	77	175	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC
2996	MIRU 26	51	285	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG(VIC)
802	MIRU 40	54	354	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	53	482	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT (FAM)
1644	MIRU 16	53	565	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC(VIC)
3192	MIRU 31	53	492	ACTGATTGGCTTCATACGGCTTA GTGCCGACGTGGTCTTGAT (NED)
424	Mtub 04	51	537	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC (FAM)
577	ETR-C	58	171	CGAGAGTGGCAGTGGCGGTTATCT(VIC) AATGACTTGAACGCGCAAATTGTGA
2165	ETR A	75	195	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT
2401	Mtub 30	58	247	CTTGAAGCCCCGGTCTCATCTGT (FAM) ACTTGAACCCCCACGCCCATTAGTA
3690	Mtub 39	58	272	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
4156	QUB-4156	59	563	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT(NED)
2163b	QUB-11b	69	77	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT(FAM)

1955	Mtub 21	57	92	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT(FAM)
4052	QUB 26	111	187	AACGCTCAGCTGTCGGAT (NED) CGGCCGTGCCGGCCAGGTCCTTCCCGAT
154	MIRU 2	53	402	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT(FAM)
2531	MIRU 23	53	150	CTGTCGATGGCCGCAACAAAACG(VIC) AGCTCAACGGGTTCGCCCTTTTGTC
4348	MIRU39	53	540	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCAACACAT(NED)
2059	MIRU 20	77	437	TCGGAGAGATGCCCTTCGAGTTAG(FAM) GGAGACCGCGACCAGGTACTTGTA
2687	MIRU 24	54	395	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA(VIC)
3007	MIRU 27	53	498	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA(NED)
2347	Mtub 29	57	335	GCCAGCCGCCGTGCATAAACCT(FAM) AGCCACCCGGTGTGCCTTGTATGAC
2461	ETRA B	57	347	ATGGCCACCCGATACCGCTTCAGT(VIC) CGACGGGCCATCTTGATCAGCTAC
3171	Mtub 34	54	326	GGTGCGCACCTGCTCCAGATAA(NED) GGCTCTCATTGCTGGAGGGTTGTAC

Appendix II. Papers and manuscripts generated from this PhD thesis

Zufan Bedewi, Adane Worku, Yalemtsehay Mekonnen, Getnet Yimer, Girmay Medhin,Gezahegne Mamo, Rembert Pieper and Gobena Ameni Molecular typing of *Mycobacterium tuberculosis* complex Isolated from pulmonary tuberculosis patients in central Ethiopia. Submitted.

Zufan Bedewi, Yalemtsehay Mekonnen, Adane Worku, Girmay Medhin, Aboma Zewde Getnet Yimer, Rembert Pieper, and Gobena Ameni. *Mycobacterium tuberculosis* in central Ethiopia: drug sensitivity patterns and its association with genotype. Submitted.

Zufan Bedewi, Yalemtsehay Mekonnen, Adane Worku, Aboma Zewde, Girmay Medhin, Temesgen Mohammed , Rembert Pieper and Gobena Ameni . Evaluation of the GenoType MTBDR*plus* Assay for Detection of Rifampicin and Isoniazid Resistant *Mycobacterium tuberculosis* Isolates in Central Ethiopia. Submitted.