

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH
SCIENCE, SCHOOL OF MEDICINE, DEPARTMENT OF
MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY



MOLECULAR EPIDEMIOLOGY AND DRUG SENSITIVITY OF
MYCOBACTERIUM TUBERCULOSIS ISOLATES AMONG NEW
PULMONARY TUBERCULOSIS PATIENTS IN ARSI, OROMIYA REGION,
ETHIOPIA

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Abbreviations

AFB.....	Acid Fast Bacilli
AIDS.....	Acquired Immunodeficiency Syndrome
ALIBP.....	Aklilu Lemma Institute of Pathobiology
Arg.....	Arginine
ARL.....	Adama Regional Laboratory
BCG	Bacille Calmette Guerin
CD4/CD8.....	Cluster differentiation 4/8
CDC.....	Center for Disease Control
DNA.....	Deoxyribonucleic Acid
DOT.....	Directly observed treatment
DST.....	Drug susceptibility testing
EDTA	Ethylene diamine tetra acetic acid
ENTPR.....	Ethiopia National TB prevalence report
EPI.....	Expansion program on immunization
ESAT-6.....	Early secreted antigen type 6
FDA.....	Food and drug administer
FLD.....	First line drug
GTR.....	Global tuberculosis report
HGT.....	Horizontal genetic transfer
HIV	Human Immunodeficiency Virus
IGRA.....	Interferon gamma release assay
ISs	Insertion sequences
LED.....	Light emitted diiodod
LJ.....	Löwenstein-Jensen

LPA.....	Line probe Assay
LTBI.....	Latent tuberculosis infection
MDR-TB	Multi-drug resistant TB
MtbC.....	Mycobacterium Tuberculosis Complex
NAP.....	Nitro alpha acetylamine
PCR.....	Polymerase Chain Reaction
PPD	Purified protein derivative
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
RFLP.....	Restriction fragment length polymorphism
RIF.....	Rifampicin
rRNA.....	ribosomal Ribonucleic Acid
RT-PCR.....	Real time polymerase chain reaction
RvD	Related Deletion
SNNP.....	South nation and national people
TB.....	Tuberculosis
TBD ₁	M. tuberculosis specific deletion
TBD1.....	Mycobacterium tuberculosis specific deletion
WHO	World Health Organization
XDR-TB.....	Extensively-drug resist

Abstract

Back ground: - The 22 high burden countries accounts 80% of global tuberculosis burden, and Africa alone account 28% of Global TB burden where Ethiopia is at 16th from the world, and 9th from the continent accounting an incidence of 200 cases per 100,000 population according to WHO 2015 Tuberculosis report. According to WHO Global TB report, Ethiopia is ranked 15th among 27 high burden MDRTB and XDR-TB countries. The estimated MDR-TB from new cases was 3.3% according to WHOGTR 2015. Specifically in Arsi Zone, PTB+ case notification in the past fifteen years, from 1997 to 2011 increased from 6.9 to 63 per 100,000 populations.

Objective: The objective of this study was to characterize the diversity of *Mycobacterium tuberculosis* strains circulating and to determine the drug susceptibility of the isolates among new PTB+ patients in Arsi zone, Oromiya region, Ethiopia.

Methods: - A cross-sectional study design using quota sampling technique was under taken. All smear positive samples were collected from seven selected health facilities which were stated having higher prevalence TB cases in the zone and parallely, questionnaire was filled for socio demographic analysis. Culture on conventional Löwenstein-Jensen (LJ) media and drug sensitivity test using Line Probe Assay (LPA) was done at Adama Regional Laboratory (ARL). Spoligotyping was done in Aklilu Lemma Institute of Pathobiology (ALIPB), AAU and the isolates were compared with the SpoIDB4 database of the Pasteur Institute of Guadeloupe.

Results: A total of 150 smear positive pulmonary TB patients were included in this study of which 130 samples were grown on LJ media. The study participant's age range was from 6 -72 years with the mean of 29 and (\pm SD) of 13.25, of which 10 % (15/150) of the study participants were below the age of 15. Molecular characterization showed that 104/130(80.0%) were Euro-American, 15/130 (11.5%) Indo-Oceanic, 9/130 (6.9%) Unknown lineage, 1/130(0.8%) West African-1 and 1/130(0.8%) *Mycobacterium bovis*. Drug Sensitivity Test on Line Probe Assay showed that 2 (1.53%) isolates were mono resistance for Rifampicin, 5 (3.8%) mono resistance for INH, and 3 (2.3%) were resistant for both INH and Rifampicin. Prevalence of MDR from New patients was 5/130 (3.8%).

Conclusion: - In this study we have shown that the prevalence of MDR-TB from the new TB patients is 3.8% which is high, and the type of the TB lineage circulating dominantly is Euro-America from which the family of "T" is the most circulating strain indicating that there is a rapid circulation among the society.

Keywords: - *Mycobacterium tuberculosis*, Molecular epidemiology, DST, spoligotyping,

Chapter One

Introduction

1.1. Background

Samples collected from Pre Columbian and Egyptian mummies indicated the presence of TB about 5,000 years ago, virtually now adays present all over the world. Previously it has had different naming's the so called white plague (reflecting the paleness of TB patients), consumption (TB patients literally consumed from their inside) and wasting disease. TB has possibly caused more human deaths than any other microbe. (Martin Spiess, 2012)

Tuberculosis is a disease of broad host range, caused mainly by more than seven different species with common name of Mycobacterium tuberculosis complex (MTC) and remain public health problem (Aranaz *et al.*, 1999). Despite the presence of modern therapy it is showing increment each year, thus, in 2011 there were a total of 8.7 million (13% co-infected with HIV) new cases reported globally and the deaths from TB were 1.4 million, by the other year, in 2013, 9.0 million new TB cases and 1.5 million TB deaths were reported, of those 1.1 million were HIV-negative and 0.4 million were HIV-positive people. (WHO, 2012, WHO, 2014)

Geographically Asia and Africa are the highest TB burden continents. Most of the estimated number of cases in 2014 occurred in Asia (58%) and the African Region (28%) with the list Region of the Americas accounts (3%). Approximately one quarter (29%) of the world's cases is from Africa. Ethiopia is being among the high TB burden countries. According to WHO global estimate of 2014, there was 126 TB cases per 100 000 population. The absolute number of incident cases is falling slowly at an average rate of 1.5% per year 2000–2013 and 0.6% between 2012 and 2013. (WHO, 2015)

The six countries that stand as having the largest number of incident cases in 2014 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). India and China alone accounted for 24% and 11% of global cases respectively. Of the 9.0 million cases 550,000 were children and 3.3 million occurred among women. (WHO GTR, 2015). In addition,

male sex, history of asthma, adult overcrowding and family history of TB are additional risk factors. (Lienhardt *et al.*, 2005)

South African reports to the World Health Organization (WHO) indicate that tuberculosis (TB) notifications have increased fivefold over the last 20 years in 2008. South Africa had the third-highest TB burden after India and China. South Africa and Swaziland now have the highest TB notification rates in the world with about 1% of their populations developing TB annually. South Africa was responsible for approximately 25% of the global burden of HIV associated TB cases in 2007 (Wood *et al.*, 2011).

According to WHO 2015 report for Ethiopia, from the total population of 94,101,000 there were an average prevalence of 190/100,000 population and an average incidence rate of 200/100,000 in which the incidence rate for HIV positive individuals is going to be 11 fold, having the mortality rate of 5.9. (WHO, 2015).

According to the 2011 national TB survey result, the prevalence of all forms of TB was 240 and PTB+ was 108 per 100,000 population (ENTPR 2011). However studies from northern, southern and central Ethiopia, the prevalence of smear PTB+ ranged from 76 to 189 per 100,000 population suggesting TB prevalence varied across different geographical locations of the country. Moreover evidences from northern Ethiopia and other African counties have shown there is clustering of TB cases and variation in TB prevalence rate across different geographic settings (Firdessa, *et al.*, 2013).

As the research done in Oromiya region Jimma Zone Agaro teaching health center south west Ethiopia showed, the overall five years prevalence of smear positive pulmonary tuberculosis was found out to be 10.9%. (Hussien A. *et al.*, 2012).

All forms of TB and smear positive pulmonary TB (PTB+) case notification increased from 14.3 to 150 per 100,000 population with an increment of 90.4% in fifteen years in Arsi Zone Oromia Ethiopia. Similarly PTB+ case notification increased from 6.9 to 63 per 100,000 populations an increment of 89% in fifteen years. The fifteen-year average TB case notification of all forms varied from 60.2 to 636 (95% CI: 97 to 127, $P < 0.001$) and PTB+ from 10.9 to 163 per 100,000 population (95% CI: 39 to 71, $p < 0.001$) in the 25 districts of the zone. (Hamusse *et al.*, 2014)

Mycobacteria that cause tuberculosis in mammals form the *Mycobacterium tuberculosis* complex (MtbC) and include *Mycobacterium tuberculosis*, *Mycobacterium africanum* which is divided into subtype I of having group A for West African (“*M. bovis*-like”) and subtype II of having group B for East African (“*M. tuberculosis*-like”), *Mycobacterium bovis* along with the *M. bovis*-derived bacillus Calmette-Gue’rin [BCG] vaccine strains, *Mycobacterium microti*, *Mycobacterium bovis* subspecies caprae (*M. caprae*), and “*Mycobacterium tuberculosis* subspecies canettii” (“*M. canettii*”). (Richard *et al.*, 2003)

Research done among children found in Jimma zone, from 101 samples collected 32 (32/101) of the samples were positive for AFB by microscopy, culture and PCR. Out of 25 AFB isolates 60% (15/25) were identified as *M. tuberculosis* by PCR, and 40% isolates (10/25) were confirmed to be non-tuberculosis mycobacteria (NTM) by genus typing and 16S rDNA gene sequencing. Lineage classification assigned the *M. tuberculosis* strains into Euro-American (EUA, 66.7%), East-African Indian (EAI; 13.3%), East-Asian (EA; 6.7%) and Indio-Oceanic (IO; 6.7%) lineages, and three isolates were new to the SpolDB4 database (Workalemahu *et al.*, 2013)

Drug resistant tuberculosis commonly arises through the selection of mutated strains by inadequate chemotherapy. Resistance to at least the two major anti-tuberculosis drugs isoniazid and rifampicin has been termed as multidrug-resistant tuberculosis (MDR-TB). Treatment of MDRTB requires prolonged and expensive chemotherapy using second line drugs of heightened toxicity. (FMOH; 2009)

About 3.5% of new tuberculosis (TB) patients in the world have multidrug-resistant strains (MDRTB). Levels are much higher about 20.5% in those previously treated for TB. The frequency of MDR-TB varies substantially between countries. About 9% of MDR-TB cases are also resistant to the two most important second-line drugs classes to extensively drug-resistant TB (XDR-TB). By September 2013, 92 countries had reported at least one XDR-TB case. (WHO GTR. 2014)

WHO estimates that there were about 480,000 new MDR-TB cases on the world in 2013. More than one half of these cases occurred in China, India, and the Russian Federation. About 210,000 MDR-TB deaths are estimated to have occurred in 2013. And the treatment success rate was 48% in 2012 but five of the 27 high MDR-TB burden countries including Ethiopia had achieved a treatment success rate of greeter than 70%. (WHO 2012 & 2014)

Multi drug-resistant tuberculosis (MDR-TB), being resistance to at least rifampicin and isoniazid was found in 5% of new cases and 24% of previously treated patients. The major genotype observed was of the Central Asia spoligotype family (CAS1_Delhi), representing 49% of the 232 isolates examined. (Sharaf Eldin *et al.*, 2011).

The overall prevalence of drug resistance to at least a single drug was 77/230(33.5%) in Eastern Amhara Regional State Ethiopia. The prevalence of MDR-TB in all, new and re-treated patients was 15/230(6.5%), 3/165(1.8%) and 12/65 (18.5%) respectively. (Esmael *et al.*, 2014).

According to WHO definition extensively drug-resistant TB (XDR-TB) is a form of TB resistant to all the most effective drugs (i.e. MDR-TB plus resistance to any fluoroquinolone and any of the second-line anti-TB injectable drugs: amikacin, kanamycin or capreomycin). That is much more difficult to treat and it has been described in 2006. Proportions of XDR-TB among MDR-TB range from 4% in Armenia to almost 24% in Estonia. But the average percentage of XDR-TB from MDR-TB is 9%. (GTR 2011, WHO 2014).

Of 544 Patients who were positive for HIV with a median CD4 of 63cells/ μ l and TB culture positive, 41% were found to have MDR TB thus 24% of those MDR patients were found to have XDR TB and 98% death among these. This may tell us the presence of retroviral infection may increase the incidence of XDR-TB when compared with HIV negative individual. (Shenoia *et al.*, 2009).

The research done in three different parts of Ethiopia- Bahirdar, Fitcha and Ambo showed that the overall proportion of MDR was 11.8% and highest (17.6%) at Ambo (Western Shoa, Ethiopia) while (8.3%) isolate out of the 11.8% MDR –TB was found XDR and was in Bahirdar. (Hussien *et al.*, 2013)

1.2. Literature review

1.2.1. Mycobacterium tuberculosis complex (MtbC)

Mycobacterium tuberculosis, *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti* are the classical members of the M. tuberculosis complex ((MtbC) or (MTC)). These pathogens which affect both humans and animals are closely related from a taxonomic point of view. Some phenotypic characteristics have been used for speciation within the M. tuberculosis complex. The fact that these groups of organisms are important as pathogens of worldwide significance has led to bias in the selection of tests for the classification of the mycobacteria. In relatively recent times, because of the increased research on tuberculosis and availability of new molecular tools other isolates with characteristics of the *M. tuberculosis* complex but not matching any of the classical species have been described, i.e. '*M. canettii*'. Despite the fact that those bacteria which show relatively similar characteristics with Mycobacterium tuberculosis complex are taxonomically classified as Mycobacterium tuberculosis subspecies caprae. (Aranaz *et al.*, 1999)

Despite their diversity in terms of their host tropism, metabolism, growth, environmental niche, epidemiology and pathogenicity, the group has 99.9% similarity at nucleotide level and identical 16s RNA sequence (Huard *et al.*, 2003). The genotypic grouping of MTC after sequencing revealed that only two loci KatG codon 463 CTG (Leu) and gyrA codon 95A ACC (Thr) were present at high frequency. Accordingly based on the combination of polymorphism located at these sites all isolates of *M. africanum*, *M. bovis* and *M.canetti* had the characteristics of group 1 (KatG463 CTG (Leu) and gyrA95 ACC (Thr)). Whereas *M. tuberculosis* in addition to group1 fell in to group 2 and 3 KatG 463 CGG (Arg) and gyrA95 ACC (Thr) and KatG463 CGG (Arg) and gyrA95 AGC (Ser) respectively (Sreevatsan *et al.*, 1997).

The member of MTC includes the primary causative agent of TB, *M. tuberculosis*, the main causative agent of TB in West Africa, *M africanum* and *M. bovis* including the vaccine strain *M.bovis* BCG which is responsible for bovine TB (Kallenius *et al.*, 1999). The others uncommon in human are *M. microti* a pathogen of voles and rarely infecting humans. *M. canettii*, are MTC strain that produces smooth and glossy colonies with all known cases so far isolated from individuals from the horn of Africa. *M. pinippedii*, also known as the seal bacillus and *M. caprae*, primarily isolated from goats. (Cousins *et al.*, 2003).

1.2.2. Evolution and Epidemiology of tuberculosis

Long time ago there was a belief that *M. bovis* is the origin for *M. tuberculosis* but finally disproved by sequencing the whole genome of *M. tuberculosis* strain (H37Rv) and *M. bovis* strain (BCG) to look for the presence or absence of Direct repeats (DRs), H37Rv related deletions (RvD) and specific deletion 1 (tbD1) in species of mycobacterium tuberculosis. The analysis of complete genome of *M. bovis* confirmed that there were no gene clusters that are confined exactly to *M. tuberculosis*. Presence or absence of *M. tuberculosis* specific deletion (tbD1) is the base for the division of *M. tuberculosis* strains into modern from ancestral strain. (Fleischmann *et al.*, 2002)

The distribution of 20 variable regions resulting from insertion deletion events in the genomes of the tubercle bacilli has been evaluated in a total of 100 strains of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium microti*, and *Mycobacterium bovis*. This approach showed that the majority of these polymorphisms did not occur independently in the different strains of the *M. tuberculosis* complex but resulted from ancient irreversible genetic events in common progenitor strains. Based on the presence or absence of *M. tuberculosis* specific deletion (TbD1), *M. tuberculosis* strains can be divided into ancestral and modern strains. (Brosch *et al.*, 2002)

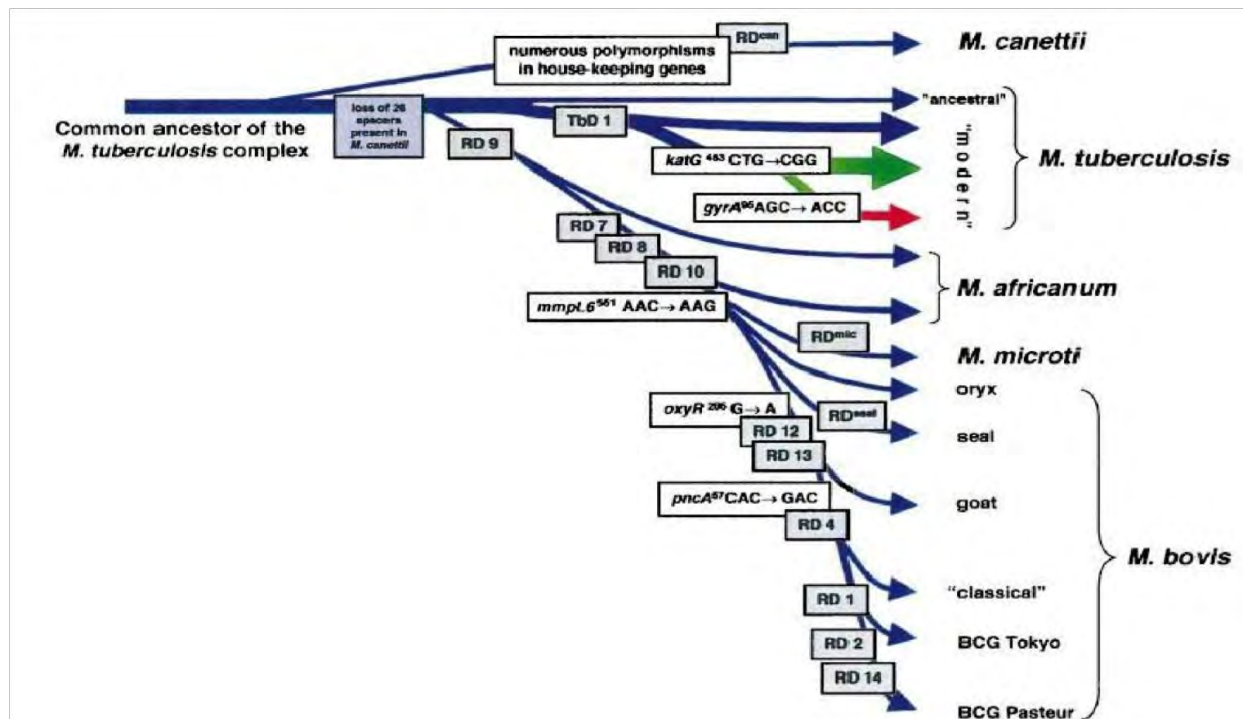


Figure 1:- Evolutionary Pathway of Tubercle Bacilli, (Brosch *et al.*, 2002)

1.2.3. Drug resistant tuberculosis

The incidence of drug resistant tuberculosis around the world has been poorly defined until recently when the World Health Organization and the International Union against Tuberculosis and Lung Disease completed a global surveillance project on drug resistance.

Globally in 2013 an estimated 480 000 people developed multidrug-resistant TB (MDR-TB) and there were an estimated 210 000 deaths from MDR-TB. Progress in the detection of drug-resistant TB has been facilitated by the use of new rapid diagnostics. According to WHO 2014 report a total of 97,000 patients were started MDR-TB treatment in 2013. However, 39,000 patients were on waiting lists and the gap between diagnosis and treatment widened between 2012 and 2013 in several countries. Extensively drug-resistant TB (XDR-TB) has been reported by 100 countries in 2013. On average an estimated 9% of people with MDR-TB have XDR-TB. (WHO 2014)

According to WHO 2010 profile on MDR-TB in Kenya from 817 cases suspected and tested for susceptibility by that year 112(13.7%) patients were categorized to MDR-TB, from which 103 of 107 were re-treatment cases and 4 patients were new cases. (WHO 2010)

Different reports in Ethiopia have shown that drug resistance is becoming increasing. MDR-TB was reported as 2% in samples taken from Addis Ababa and Harar (Wolde *et al.*, 1986). In 1997, 0.4% of new patients from Harar (Mitike *et al.*, 1997) and 1.2% of new TB patients from Addis Ababa (Demissie *et al.*, 1997) were reported to have MDR TB. Recently the percentage of MDR from new case goes to 3.5% according to (WHO 2015).

On the other hand according to the research done in Ethiopia Amhara Region to know the prevalence of MDR-TB from the total of 606 sample size, the overall prevalence of MDR-TB cases was 93(15.3%). Rifampicin (RMP) and isoniazid (INH) mono resistance were 17(2.8%) and 15(2.5%) respectively. Considering RMP mono resistance as surrogate marker for MDR TB, prevalence of MDR TB/RMP resistance was 110(18.2%). Moreover, the rate of MDR TB among smear and/or culture positive samples were 42.9% and together with RMP mono resistance, it increased to 50.7%. (Debebe *et al.*, 2013).

1.2.4. Natural Course of Tuberculosis

Airborne droplets containing (1-5 μ m) *M. tuberculosis* are inhaled and can reach the pulmonary alveoli then infects and replicate within the alveolar macrophage. A granulomatous lesion can develop in which if the host is immune-competent to contain and limit the mycobacteria. Within a granuloma in which there is a highly heterogeneous immune cell population containing a variety of different T-cell populations, B-cells, macrophages at different stages of maturation, and dendritic cells. If the cellular immune response is altered or debilitated by any other conditions then the granuloma may no longer be able to contain and limit spread of the mycobacteria. The granuloma liquefies and mycobacteria are released not only within the lung but can disseminate to other extra-pulmonary sites via the lymphatic system and blood resulting in disseminated disease known as extra-pulmonary tuberculosis. (Kaufmann *et al.*, 2008)

Despite the apparent strong immune response which contains the *M. tuberculosis* infection in the granuloma, the bacilli are yet not fully eliminated. Ultimately latent TB converts into active disease when cavitory lesions develop and the number of bacilli increases in the caseous center of the granuloma. Live bacilli can reach the alveoli once the granuloma center collapses and the patient becomes infectious. Viable and infectious bacilli are freed into the airways resulting in a productive cough spreading infectious bacilli in the air. (Barry *et al.*, 2009)

The life cycle continues when other persons get infected. It is estimated that a single person with active TB can infect up to 45 other individuals. An individual infected and developing active pulmonary TB shows symptoms like prolonged and productive cough duration of greater than two weeks, shortness of breath, chest pain, night sweats, weight loss, or fever. In adults, active TB typically results from the reactivation of existing latent TB rather than as direct outcome of primary infection. Generally the immediate onset of disease following infection only occurs in immune-compromised individuals such as newborns, elderly or HIV infected patients. In fact the conditions influencing the progression to active TB are not fully understood, although factors such as HIV and diabetes are known to favor a prompter development of active tuberculosis (Vynnycky *et al.*, 2001).

1.2.5. Genomic Variability of *Mycobacterium tuberculosis*

In the clinical setting, genomic variability can represent a significant barrier to treatment. Many pathogens can acquire mutations or foreign genetic material through horizontal gene transfer (HGT) in response to the selective pressure imposed by the host immune system and by chemotherapy resulting in strains that are difficult to eradicate in hospitals as well as during long term infection. Understanding the extent of genomic variability and its effects on disease in the case of pathogens that display genetic homogeneity and low variability as the case for the causative agent of tuberculosis, *Mycobacterium tuberculosis* is particularly fascinating. (Zambrano *et al.*, 2012)

Phylogenetic studies have also shown that clinical strains of *M. tuberculosis* are more genetically variable than originally expected. Moreover genetic variability can be translated into phenotypic differences such as transmission capacity, virulence and pathogenicity that can have epidemiological consequences and affect the outcome of the disease. (Dyamoya *et al.*, 2011) *M. tuberculosis* has spread globally more than any other lineage (Barletta *et al.*, 2013)

In addition to transmission capacity it is also currently accepted that genetically different *M. tuberculosis* strains produce markedly different immuno-pathological events and affect disease manifestation. For example in a study conducted in Vietnamese patients a clear association between the Euro American Lineages of *M. tuberculosis* and pulmonary rather than meningeal tuberculosis was observed suggesting these strains are less capable of extra-pulmonary dissemination than other strains in the study population. (Caws *et al.*, 2008)

Isolates can be further characterized by PCR-RFLP analysis of the oxy-R gene to differentiate between *Mycobacterium bovis* and *M. tuberculosis* within the MTB complex doing this procedure will help to know the typical strain in a given population. Research done in Egyptian patients showing that isolates contained IS6110 based up on PCR amplification of a 123-bp region of the insertion element among the 45 human isolates, one was determined to be *M. bovis* and 44 were *M. tuberculosis* based on amplification of a 270-bp region of oxy-R and when the 40 RFLP patterns were compared with those data base containing more than 6000 distinct patterns at the Center for Disease Control and Prevention, only one pattern found to be similar and this isolate found to be multi drug resistance MDR TB. (Abbadi *et al.*, 2009)

The research done in Kenya Nairobi to show the Diversity of Mycobacterium tuberculosis strains in their country, different strain families were identified from 536 isolates. The principal groups were looks like; CAS1_KILI 96/536 (17%), T1 69/536 (12%), Beijing 65/536 (12%), LAM9 46/536 (9%), LAM3 37/536 (7%), LAM11_ZWE 26/536 (5%), CAS1_DELHI 24/536 (4%) and T2 24/536 (4%). New *M.tuberculosis* strain family were also identified with 21/536 (4%) in which they designated as Nairobi subtype and those they identified are not previously included in the SpolDB4 accounted for 15/536 (3%). (Ogaro D. *et al.*, 2012)

Ferdessa *et al.*, on his project for determination of isolates taken from different parts of Ethiopia found that most of his isolates had an intact RD9 region which identified them as *M. tuberculosis*. Only 4/964 (0.4%) of isolates had undergone RD9 and RD4 deletions characteristic of *M. bovis*. The 4 *M. bovis* isolates were obtained from cases of pulmonary TB. And also 10 non mycobacterial tuberculosis isolates were identified as *M. intracellulare*, *M. flavescens*, and *M. simiae*. Thus the principal investigator conclude that the overall contribution of *M. bovis* to human TB is minor but greater in specific areas. In Ethiopia, monitoring of zoonotic transmission is needed in urban areas with high rates of bovine TB associated with intensive farming of imported dairy cattle. (Firdessa *et al.*, 2013).

1.2.6. History of Tuberculosis Treatment and Drug Resistance

Drug resistance is defined as a decrease in susceptibility of sufficient degree from a wild strain that has never been exposed to the drug (WHO 1997). Generally when one percent or more of organisms in an isolate are found resistant to an anti-tuberculosis drug therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Rom and Garay, 1996). Normally any large population of Tuberculosis bacteria regardless of their exposure to antibiotics will contain some organisms resistant to one of the five first line drugs, isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide. (Selwyn *et al.*, 1992).

Drug resistance is a natural phenomenon and could occur at any time during bacterial replication. It arises from random mutations of the bacterial chromosome that occur spontaneously in wild type strains. When mutation confers resistance to a certain antibiotic, all sensitive bacteria are killed and the resistant ones will grow and become the dominant variant in the population (Porter and Mc. Adam, 1992).

Although drug resistant strains must have existed before the development of antibiotics. Their frequency of occurrence increased after the development of anti-tuberculosis drugs in the 1950s (Rom and Garay 1996). Drug resistance in tuberculosis could therefore occur in naive patients who have never been treated with any anti tuberculosis drugs i.e. primary resistance or in patients who get inadequate treatment due to prescription error or non-adherence to the appropriate regimen (WHO, 1994).

1.2.7. Diagnosis of Tuberculosis

Even though slow growing nature and fastidiousness of Mycobacterium tuberculosis complex (MtbC) are the challenges for diagnosing, there are limited diagnosing techniques that can be performed from routine laboratory to highly advanced one. These are Smear microscopy, Chest radiography, Mantoux test. In vitro culture, Molecular diagnostic and Biochemical test.

1.2.7.1. Smear microscopy

The most widely used diagnosis for active pulmonary TB is the examination of sputum smear by microscopy after ZiehlNeelsen and Florecense staining. The ZiehlNeelsen staining method relies on the specific lipid rich cell wall of mycobacteria which retains the red carbolfuchsin (the first dye on staining) dye after alcohol acid de-colorization. This is the reason why *M.tuberculosis* is often referred as “acid fast bacillus” depicts the structure of the cell wall. (Barry *et al.*, 2009)

Sputum smear examination by light microscopy is a highly specific, fast and cheap method and often the only TB diagnosis available especially in resource limited settings. Unfortunately, the sensitivity of smear microscopy is variable with 20 to 80%, *M. tuberculosis* infections missed or underdiagnose by this method. Apart from the low sensitivity, the main drawback of sputum smear microscopy is the intrinsic reliance on sputum production limiting its use in children and HIV positive patients which can't expectorate and give proper sample. Fluorescent microscopy is a faster, more sensitive but less specific alternative using acid fast fluorochrome dyes instead of ZiehlNeelsen staining. In 2011, WHO recommended to initiate the implementation of fluorescent microscopy with a light emitting diode (LED) for the diagnosis of TB. (Truffot *et al.*, 2006 and Davies 2008)

1.2.7.2. Chest radiography

Chest radiography (X-rays) is often used as a complement to smear microscopy to diagnose TB especially in sputum negative patients. Typical TB related abnormalities in the lungs like infiltrations, nodules or cavities can be recognized by trained medical personal but are generally not used as the only definite TB diagnosis mechanism alone. (WHO 2011)

1.2.7.3. Mantoux test

Robert Koch in 1908 described a delayed type hypersensitivity reaction to extracts from Mycobacteria cultures first in the guinea pig animal model and subsequently in humans and proposed it as an option for TB treatment. Purified extract of *M. tuberculosis* proteins now known as PPD which is now routinely used for diagnosis of *M. tuberculosis* infection by intradermal injection. The delayed type hypersensitivity response (measured as induration at the site of injection) that ensues if sensitized T cells are present is seen in those infected with *M. tuberculosis*. Thus the tuberculin skin test (TST) was the first immunodiagnostic based on an acquired immune response towards *M. tuberculosis*. The underlying mechanism is the strong cellular immune response elicited by infecting *M. tuberculosis* mediated through macrophages CD4+ and CD8+ T-cells and regulated by cytokines such as tumor necrosis alpha and interferon gamma. This response is known to be dominated by interferon (IFN- γ)-gamma production .while the size of the reaction can't discriminate the latent tuberculosis infection (LTBI) from active one. (Dheda *et al.*, 2010 and Nicod 2007)

1.2.7.4. Biochemical test

The differentiation of MTC by Biochemical analyses includes colony morphology, niacin accumulation test, growth in the presence of thiophen-2-carboxylic acid hydrazide (TCH; 2 μ g/ml), nitrate reduction on modified Dubos broth, and growth characteristics on Lebek medium and on bromcresol purple medium (induction of a pH-dependent change of color from blue to yellow). And their interpretation is: - Oxygen preference in Mycobacterium isolates on Lebek (a semisolid medium) can be described as aerophilic (growth on the surface) and micro aerophilic (growth below the surface). Nitrate reduction and niacin accumulation are the characteristics of *M.tuberculosis*. *M. bovis* is intrinsically resistant to pyrazinamide (PZA), major criteria for differentiation however susceptibility to PZA among isolates of *M. bovis* has been reported in

some studies. Biochemical tests have now been replaced with molecular techniques for the identification and classification of MTC. (Normung, 1986)

1.2.7.5. In vitro culture

Bacterial cultures in vitro can provide a definitive TB diagnosis and is currently considered as the gold standard method. Unfortunately *M. tuberculosis* grows very slowly with a doubling time of about 20 hours (depending on the growth medium used), thus it can take weeks. The sensitivity is high as only a few viable bacilli are sufficient to initiate growth of sputum samples must be decontaminated before culture inoculation to prevent overgrowth of other faster growing microorganisms. However decontamination can also be harmful to mycobacteria and therefore culture based diagnosis is not a 100% sensitive method. The main drawback of *M. tuberculosis* culture is the requirement for high biosafety laboratory facilities biosafety level 2 and 3 and specifically trained staff to avoid aerosol transmission. Such facilities are very costly and often not available in resource limited settings. (Rieder *et al.*, 2007)

1.2.7.6. PCR

The development of PCR technique in general is for detection of specific sequences of *M. tuberculosis* and other mycobacteria. This PCR assays may target either DNA or rRNA of MtbC and these could be based on conventional DNA based PCR, nested PCR and real-time (RT-PCR). Targets include insertion and repetitive elements, various protein encoding genes, ribosomal rRNA, etc. Developments in this area have been very rapid and a large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described. In general, gene amplification methods have been found to be highly sensitive and specific for diagnosis of tuberculosis directly from clinical specimens. Depending upon the bacteriological status and copy number of target sequence, sensitivity has ranged from (70-100%) whereas specificity between 80-100%. (Katoch 2004)

1.2.7.7. Interferon Gamma Release Assay (IGRA)

Interferon-Gamma Release Assays (IGRAs) are whole-blood tests that can aid in diagnosing *Mycobacterium tuberculosis* infection. But they do not help to differentiate latent tuberculosis infection from active tuberculosis disease. There are two IGRAs techniques that have been approved by the U.S. Food and Drug Administration (FDA) and are commercially available.

They are Quantiferon – TB gold in-tube test (QFT–GIT) and Spot-TB test (T–Spot). To look for how it work, IGRAs measure a person’s immune reactivity to *M. tuberculosis*. White blood cells from persons that have been infected with *M. tuberculosis* will release interferon-gamma (IFN-g) when mixed with antigens (substances that can produce an immune response) derived from *M. tuberculosis*. To conduct the tests, fresh blood samples has to be mixed with antigens and controls. The antigens testing methods and interpretation criteria for IGRA is differ. The advantages of IGRA is patient visit to conduct the test results can be available within 24 hours and BCG vaccination does not cause a false-positive test result. The disadvantages and limitations of IGRAs is, blood samples must be processed within (8-30) hours after collection while white blood cells are still viable and errors in collecting or transporting blood specimens can decrease the accuracy of IGRAs. (CDC guideline for IGRA)

1.2.7.8. Spacer-Oligotyping (Spoligotyping)

Spoligotyping is a new method for simultaneous detection and typing of MTC. This method is based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat (DR) locus in the *M. tuberculosis* genome. Results can be obtained from *M. tuberculosis* culture within 1 day. Thus the clinical usefulness of spoligotyping is determined by its rapidity both in detecting causative bacteria and in providing epidemiologic information on strain identities. Implementing such a method in clinic settings would be useful in surveillance of tuberculosis transmission and in interventions to prevent further spread of this disease but it wouldn’t be easy to implement in resource limited setup. (Gori *et al.*, 2005)

1.2.8. Epidemiology of Drug Resistance Tuberculosis

Drug-resistant and multidrug-resistant (MDR-TB) resistant to at least isoniazid (INH) and rifampin (RMP) strains of *Mycobacterium tuberculosis* are seen to be man-made problems, mainly related to poor case management and lack of quality drugs. *M. tuberculosis* complex use several strategies to resist the action of antimicrobial agents (WHO 2009). Eighty five percent of MDR-TB occurs in 27 countries (WHO 2011). Globally, 3.5% of new cases and 20% of previously treated cases are estimated to have MDR-TB. Ethiopian national drug resistance survey of 2005 estimated the prevalence of MDR-TB among new and retreatment cases was 1.6% and 12% respectively (WHO 2011).

According to Abate *et al.*, research done at St.Peter's TB Specialized Hospital, of 376 TB isolates 102 (27.1%) were susceptible to all of the four first line anti-TB drugs -Isoniazid, Rifampicin, Ethambutol & Streptomycin, and 274 (72.9%) were resistance to at least one drug. And the individual drug resistance pattern looks like: - STM (67.3%) was found to be the most common followed by (56.1%) INH, (46.1%) RIF and (43.5%) ETB. And also there were no mono resistant strain for Rifampicin. And poly resistance was reported in 29 (7.7%) of the cases. Among these, the highest proportion was 15(53.6%) (Isoniazid and Streptomycin) combination. In this study the prevalence of MDR-TB (defined as the resistance to at least INH and RIF) was 174 (46.3%). (Abate *et al.*, 2012)

The research done in three different parts of Ethiopia (Bahirdar, Fitcha and Ambo) showed that the overall proportion of MDR isolates was 11.8% and highest (17.6%) at Ambo (Western Shoa, Ethiopia) while one (8.3%) isolate out of the 11.8% MDR M. tuberculosis was XDR and was found in Bahirdar (North-West Ethiopia). (Hussien *et al.*, 2013),

1.2.9. Diagnosis of Drug resistant Tuberculosis

Diagnosis of drug resistance TB is one of the essential steps in the management of tuberculosis. To meet this requirement several methods have been developed, these are conventional (absolute concentration, resistance ratio and proportion), BACTEC, MIGT, molecular methods etc. (Mshana *et al.*, 1998). However these methods have a number of limitations. More rapid methods like BACTEC are very expensive for routine use in high TB endemic and low economic countries. Conventional methods that use solid media (Lowenstein-Jensen media or 7H10 Middlebrook agar) take 6 to 9 weeks to obtain the result. Therefore simple, rapid and relatively inexpensive methods are desired particularly for low-income countries. (Foonglada *et al.*, 2002). Recently a simple and rapid colorimetric assay has been developed. This method uses Tetrazolium salt for a reliable detection of drug resistant strains (Caviedes *et al.*, 2002).

1.2.9.1. Phenotypic Drug Susceptibility testing Methods

Susceptibility testing of mycobacteria utilizes the same solid media, broths and inoculation methods as culture techniques. The systems are supplemented with anti-tuberculosis drugs. Growth of the organisms in the presence of anti-tuberculosis drugs is compared to controls in order to interpret susceptible or resistance. According to (drug-resistant tuberculosis a survival guide for

clinicians), there are different mechanisms of performing drug susceptibility testing and looks the following:-

1.2.9.1.1. Agar proportion method: -

The clinical specimen (direct method) or a subculture of mycobacterial growth (indirect method) is used to inoculate agar plates containing either an anti-TB drug or no drug (control). The growth of colonies on the drug-containing quadrant is compared to the control quadrant as a proportion (percent resistance). This process typically takes at least 3 to 4 weeks.

1.2.9.1.2. Direct method: -

The clinical specimen (usually AFB smear-positive sputum) is processed and then inoculated directly onto agar plates containing anti-TB drugs. And the growth of Tuberculosis in the presence of anti-tuberculosis drugs will be analyzed against control run without drug.

1.2.9.1.3. Indirect method: -

After the *M. tuberculosis* grows from a clinical specimen, a suspension is prepared and inoculated onto drug-containing agar plates or into broth bottles or tubes. The result will be observed by the degree of turbidity in accordance with the control tube containing no drug.

1.2.9.1.4. Broth methods: -

A cell suspension of *M. tuberculosis* is inoculated into vials or tubes of broth containing either the critical concentration of an anti-TB drug or no drug (control). The growth of the organism in the drug-containing medium is compared to the growth in the control. Broth methods are preferred for first-line testing as they are much faster (typically 5 to 10 days) than the proportion method using agar media.

1.2.9.1.5. Newer broth methods: -

Other broth systems have been developed to detect mycobacterial growth in a fully automated system. In addition these systems can be used for drug susceptibility testing which are mentioned on (Drug-resistant tuberculosis | a survival guide for clinicians) and approved by USFDA are the following:-

1.2.9.1.6. BACTEC system

This assay system developed by Becton Dickinson is based on generation of radioactive carbon dioxide from substrate palmitic acid. This method has been extensively used all over the world and growth can be detected within 5-10 days. Inclusion of NAP (beta nitro alpha acetyl amine beta hydroxyl propiophenone) helps in distinguishing *M.tuberculosis* (inhibited) from other mycobacteria. This system has been widely used for drug susceptibility testing and is currently used as a comparative standard. (Katoch 2004). It has been validated to provide results for SM, INH, RIF, EMB (SIRE) and PZA in a time frame close to the BACTEC system. MGIT, 320, 640 and 960 has also been approved by Food and Drug Administration (FDA).

1.2.9.2. Molecular Detection of Drug resistance Tuberculosis

In this technique the DNA is extracted from the bacteria and amplified. Certain mutations associated with drug resistance can be detected. Gene X-pert and LPA are one of the Molecular techniques of detecting mutated gene which causing resistant.

1.2.9.2.1. Gene X-pert

Molecular diagnostic tests based on DNA amplification have been proposed to complement the conventional bacteriological diagnosis of TB. Most of these tests have the advantage to allow the rapid detection of *M. tuberculosis* and are highly specific although their sensitivity often remains sub-optimal. Nevertheless, newer assays are considerably more sensitive and now days a fully automated system called Xpert MTB/RIF (Cepheid-USA) has been validated in USA. Xpert MTB/RIF detects *M. tuberculosis* infections and rifampicin (RIF) resistance in approximately two hours, simultaneously from directly sputum came for smear and with high sensitivity. This method is based on polymerase chain reaction (PCR) amplifying specific regions of the (rpo-B gene). Furthermore it requires no advanced infrastructures since all reagents are available disposable cartridge that is single use. (Osman *et al.*, 2014)

1.2.9.2.2. The GenoType® MTBDRplus LPA

The GenoType® MTBDRplus LPA is based on multiplex PCR technology, followed by reverse hybridization to detect and identify *M. tuberculosis* complex and to predict the susceptibility or resistance to the two main first-line anti-tuberculosis drugs, RMP and INH. 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: *rpoB* (associated with RMP resistance) and *katG* and *inhA* (associated with high-level and low-level INH resistance respectively). Each strip contains 27 reaction zones. (FIND 2012)

1.2.10. Vaccine for Tuberculosis

In 1921 the first anti-TB vaccine was developed by Albert Calmette and Camille Guerin and named by their and the strain name called Bacille Calmette Guerin (BCG) and it remains the only available Vaccine against TB. Nowadays even though many new innovations are undergoing its efficacy against pulmonary TB in adults varies from 0 to 80%. (Brooks *et al.*, 2001)

One of the mechanisms of attenuation during the development of BCG vaccine from virulent *M. bovis* was due to the deletion of RD1 region. RD1 comprises 9 genes including early secreted antigen type 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). (Michael J. *et al.*, 2007). But now day's researchers have made significant progress in TB vaccine development and more than ten TB vaccine candidates are now being evaluated in clinical trials following two different approaches pre-exposure vaccination in order to prevent disease in individuals that have so far not encountered *M. tuberculosis* and post-exposure vaccination that aims at inhibiting disease outbreak in individuals that are already infected. (Brennan & Thole 2012)

Table 1:- Tuberculosis vaccine candidates in clinical trials. (Brennan & Thole, 2012)

No	Products	Product description	Type of vaccine	Indication	Status
1	AERAS-422	Recombinant BCG expressing mutated PfoA and overexpressing antigens 85A, 85B, and Rv3407	Recombinant Live	Prime.	Phase I
2	AdAg85A	Replication deficient adenovirus 5 vector expressing Mtb antigen 85A	Viral Vectored	Prime, Boost	Phase I
3	HyVac 4/AERAS - 404, + IC31	Adjuvanted recombinant protein composed of a fusion of Mtb antigens 85B and TB10.4	Recombinant Live	Prime, Boost	Phase I
4	Hybrid _I+CAF01	Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT_ 6	Recombinant Live	Prime, Boost	Phase I

5	Hybrid 56 + IC31	Adjuvanted recombinant protein composed of Mtb antigens 85B, ESAT_6 and Rv2660	Recombinant Live	Prime, Boost	Phase I
6	VPM 1002	rBCG Prague strain expressing listeriolysin and carries a urease deletion mutation	Recombinant Live	Prime.	Phase II
7	M72 + AS01	Recombinant protein composed of a fusion of Mtb antigens Rv1196 and Rv0125 & adjuvant AS01	Recombinant Live	Prime, Boost	Phase II
8	Hybrid I+IC31	Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT-6	Recombinant Live	Prime, Boost	Phase II
9	RUTI	Fragmented Mtb cells	Whole cell (in	Immunotherapy	Phase II

1.2.11. Treatment

Current WHO guidelines recommend a 6-month treatment regimen consisting of a combination of 4 drugs (Rifampicin, Isoniazid, Ethambutol, and Pyrazinamide) for 2 months then 2 drugs (Rifampicin and Isoniazid) for 4 months. This regimen gives curative rates of up to 90% in immunocompetent (HIV negative) TB patients. (CDC 2006). For this to be achieved this regimen has to be prescribed at the correct dose taken regularly by the patient and for the required length to time to prevent relapse and development of drug resistant strains. The drugs in the current regimens are efficient bactericidal compounds at standard or higher doses (for Ethambutol) especially for actively growing/multiplying bacteria. At lower doses or for slowly multiplying Mycobacteria (Isoniazid) they can be bacteriostatic. Pyrazinamide can be bactericidal or bacteriostatic depending on the concentration reached at the site of infection. The ability of the most commonly used TB regimens to kill the slowly growing or slowly metabolizing bacteria i.e. persists after rapidly growing or metabolizing bacteria have been killed via bactericidal activity is suboptimal. This is the main reason for the long duration of current TB treatment regimens. Generally the First line anti-TB drugs (FLD) including isoniazid, streptomycin, rifampicin and

ethambutol and there are three injectable second line drugs known as (amikacin, capromycin and kanamycin). (Zambrano *et al.*, 2012).

The most important determinant for success of therapy is the development of drug resistance. The known targets for each of the four first line drugs and the genetic location of mutations that confer drug resistance are already known, however not all mutations that confer resistance have been fully elucidated. *M. tuberculosis* isolates that are resistant to both isoniazid and rifampicin are defined as multidrug-resistant TB (MDR-TB). Resistance to isoniazid and rifampicin plus any fluoroquinolone and at least one of three injectable second-line drugs (amikacin, kanamycin, or capreomycin) is defined as extensively drug resistant TB (XDR-TB). (Shah *et al.*, 2011)

1.3. Statement of the Problem

Tuberculosis (TB) is a major global and national level, our country Ethiopia is still the member of top twenty high TB burden country having an incidence of all forms of TB was 200 per 100,000 populations with mortality rate 32 per 100,000. (WHO 2015)

Molecular epidemiology characterization has been done in different parts of the country and showed that there is diversification. Debebe *et al.* 2013, in his project showed that 36(11.1%) new strains of Mtb out of 118 samples were found and another study done by Tessema *et al.* 2013 identified 9.4% of the isolates were not found on the data base which means have no lineage classification and 45.1% of the isolate were grouped in the same cluster indicating that there is a high rate of recent transmission.

As researches has been done in the country to show the magnitude of MDR-TB. Debebe *et al.*, in his research from the total of 606 sample the overall prevalence of MDR-TB was 93(15.3%). Rifampicin (RMP) and isoniazid (INH) mono resistance were 17(2.8%) and 15(2.5%) respectively. Moreover the rate of MDR TB among smear and/or culture positive samples were 42.9% and together with RMP mono resistance, it increased to 50.7%, (Debebe *et al.*, 2013).

All forms of TB and smear positive pulmonary TB (PTB+), case notification increased from 14.3 to 150 per 100,000 population with an increment of 90.4% within fifteen years in Arsi Zone. Similarly PTB+ case notification increased from 6.9 to 63 per 100,000 populations an increment of 89% in fifteen years between September 1997 and August 2011. During their retrospective study from the total of 14,221 TB cases which were analyzed during the study period 867 (6.1%) were relapsed cases and 2,333 (16.4%) cases were treated unsuccessfully because of defaulting, failure and death. Hamusse *et al.*, (2014)

As the 15 years retrospective analysis done by (Hamusse *et al.* 2014) showed that the defaulter rate in my study area increased from (2.5 to 21.6%), which is responsible for mutation and 6.1% of the Zonal TB patients were relapse cases who have >20 % chance to be MDR-TB according to WHO 2014 estimation on retreated TB case, but when I came to my study area even though the zone is pilot site to start direct observing treatment (DOT) as national level, no research has been done to show the molecular epidemiology.

1.4. Significance of the study

Molecular epidemiology is a powerful approach for monitoring infectious diseases (Savine *et al.*, 2002). It is particularly important in the study of chronic diseases such as tuberculosis, where patients with recurrent tuberculosis can be chronically infected with a given strain and relapse due to reactivation of that strain or in contrast can be re infected by a different strain after cure (Van Rie *et al.*, 1999). A correct distinction between these alternatives is essential for accurate estimation of the success rates of tuberculosis programs (Bloom and Murray 1992). Moreover it can give unique insights into the international dissemination dynamics of *M. tuberculosis* by the comparison of isolates from different geographic areas and allows to analyze evolutionary changes of pathogen populations and strain lineages (Supply *et al.*, 2001). Molecular epidemiological results from developed countries often show high polymorphism in the genetic patterns of *M. tuberculosis* complex strains (Bauer *et al.*, 1998 and Van Solingen *et al.*, 1999).

On the other hand according to WHO 2015 report, globally 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB. This translates into an estimated 480 000 people having developed MDR-TB in 2014. On average, an estimated 9.0% of patients with MDRTB had extensively drug resistant TB (XDR-TB). According to Hamusse *et al.*, the 15 years study showed that defaulter rate grown from 2.5 to 21.6%, and death rate from 1.6 to 11.1%, across the 25 districts in Arsi zone which may be responsible for the increment of MDR and or XDR-TB

Although different studies have shown significant genetical variation among TB in the population, there is no research done to show the molecular epidemiology of Tuberculosis in Arsi zone even though the zone is the first center to be started DOT as national level in 1992. There is also no research yet done in this zone to show the MDR-TB prevalence and the resistance pattern even though the drug defaulter numbers are becoming higher especially for those re-treatment cases in the zone. Looking the possible risk factors among those pulmonary TB positive individuals will also enables the population to keep not to be infected by TB having the zone total population of 3.1 million from which about 89% of the zonal population resided in rural areas. In 2011 about 70% of the population lived within a 10-km radius or at a walking distance of 2 hours from a health institution.

Chapter Two

Objectives

2.1. General Objective

To determine the molecular diversity of the Mtb strains circulating in the area and their antibiotic susceptibility.

2. 2. Specific Objective

- To characterize the strains of mycobacterial isolates by spoligotyping
- To identify the lineage
- To study the transmission dynamics
- To determine the prevalence of MDR TB
- To determine the pattern of MDR TB

Chapter Three

Materials and Methods

3.1. Study Area

The study was conducted in Arsi zone, Oromiya region Ethiopia. Arsi is one of the zones in Oromiya Regional State. It is also one of the most densely populated zones with 148 people per km². The Zone has a population of 3,056,372 according to 2007 national census of which 1,533,610 were males and 1,522,762 were females with an area of 21,009 (km²). The zone has 25 Woreda's and each Woreda has an average of two health centers which can diagnose TB by smear microscopy. The Zone has also three hospitals and one teaching referral hospital known as Arsi University Asella teaching hospital. (BoFED, 2008)

3.2. Source Population

TB suspected Patients visited selected health facilities of the study sites (Eteya health center, Asella Teaching Hospital, Huruta Health center, Shirka health center, Kersa health center, Bekoji health center and Robe hospital) from February 2015 to August 2015.

3.3. Study Participants

All newly diagnosed individuals who became smear-positive for pulmonary TB and those who were willing to give informed consent.

3.4. Inclusion Criteria

All smear positive new pulmonary TB positive patients who were volunteers and gave informed consent, and three sputum samples (morning-spot-morning) and live in the woreda permanently.

3.5. Exclusion Criteria

Patients who were not willing to participate in this study, and couldn't give three sputum samples (morning-spot-morning); those who gave small volume (<5ml) and bloody sputum. And those who had previous history of anti TB treatment and who were not resided permanently were excluded.

3.6. Study Design and Sample Size

A cross-sectional study design was conducted, sample size was calculated by using single proportion population formula, considering prevalence of about 10.9% reported by (Hussien A. *et al.*, 2012) in Oromia region Jimma zone with 95% confidence level and 5% precision between sample and population parameter. To collect the required samples, based on calculated sample size, districts with high prevalence was purposely selected and quota sampling techniques was used in the seven selected districts.

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

Where: - n = Sample size,

$Z_{\alpha/2}$ = Z value at ($\alpha = 0.05$) = 1.96

P = Proportion of occurrence of the event to be studied (Prevalence) = 10.9%

d = Margin of error (Precision), (Usually ≤ 0.05)

Calculation; $n = 3.84 \times 0.097 / 0.0025 = 148.9$

10 % non-response rate = $148.9 \times 10/100$

= 15

N= n + 10% non-response rate

148.9 + 15

164

Table 2:- Quota sample distribution in seven selected Woredas Showing quota sample distribution in seven selected Woredas (Reference for population is from 2012 report of national population estimation based on 2007 senses

No	Woreda	Town	Population			Calculated Sampleper pop.	Expected sample	Collected samples
			M	F	Total			
1	Tiyo	Asella	41,714	41,241	82,955	73.13	73	75
2	Hetosa	Eteya	9,000	9480	18,480	16.29	16	17
3	Lode Hetosa	Huruta	8008	8350	16,358	14.4	14	11
4	Shirka	Gobessa	7,135	6,632	13,767	12.13	12	12
5	Munessa	Kersa	7,171	6,693	13,864	12.18	12	10
6	Lemuna bilbillo	Bekoji	10,890	10,988	21,878	19.28	19	15
7	Arsi Robe	Robe	10,017	8,689	18,706	16.5	17	10
Total population			93,935	92,073	186,008	164	163	150

3.7. Data collection method

Sputum samples had been collected consecutively until the required number of samples achieved from smear positive pulmonary TB patients enrolled in the study sites (Tiyo, Hetosa, Lode Hetosa, Shirka, Asasa, Munessa, and Robe Woredas). Every smear positive patient was requested to provide three sputum samples (spot-Morning-spot) and the sample was pooled in 50 ml sterile screw capped universal falcon tubes. The pooled sputum samples has been stored (4-8⁰C) for maximum of 2 weeks at each health facilities and transported in a cold chain system to the core laboratory at Adama Regional Laboratory (ARL) for culture on conventional Löwenstein-Jensen (LJ) media and isolates had been collected in 3% TSY [the mixture of 3% tryptsoya broth (TBS) and 20% Glycerol] then stored in -80⁰c at ARL, Drug susceptibility (DST) was done there in ARL by Line probe assay (LPA). Finally the isolate was transported to Akililu Lemma Institute of Patho Biology (ALIPB), Addis Ababa University (AAU) for molecular typing.

3.8. Laboratory Methods

3.8.1. Smear Microscopy

In the periphery laboratory, after the study participants were suspected for Tuberculosis by their clinicians and sent for confirmation to the laboratory, patients were directed to give productive sputum and given another screw cup plastic container to bring the morning productive sputum samples coughing from the lung in safe way, by the second day they were also giving another spot samples soon they reach the laboratory, and the three slides were investigated for the presence of TB, those became positive on smear were included for this study.

3.8.2. Sputum Culture

The pooled sputum sample was decontaminated and concentrated using NALC-NaOH The sputum samples were decontaminated and concentrated by shaking in an equal volume of NaOH-NANC for 15 minutes and concentrated by centrifugation at 3000 rpm for 15 minutes. The sediment was neutralized with 2N HCl, using phenol red as an indicator. Bacteriological culturing of sputum samples was performed using the conventional LowensteinJensen (LJ) egg slant medium, containing 0.6% sodium pyruvate and glycerol media, for the recommended time. Then the cultures were incubated at 37°C for 4-8 weeks and examined on weekly bases for the presence of any mycobacterial colonies. When visible colony was observed, acid fast staining was done to select the smear positive isolates. Isolates from the positive cultures was preserved with freezing media 3% TSY.

3.8.3. SD TB Ag MPT 64 Rapid (Capilia)

The test procedure was done, after the growth of TB seen on solid LJ media. In brief, normal saline was added to the bottom of the media and aspirated by rubber pasture pipette and dropped in to the sample pore of the Capillia cassette. Then two drops of running buffer was added. The chromatographic technique assay was interpreted as positive when red band line was seen on both control and test band indicating that it was MTC, and Negative when the red line was seen on control only indicating that the strain was NTM.



Figure 2:- Capilia tests showing an SD TB Ag MPT 64 Rapid tests

3.8.4. DST by GenoType® MTBDRplus LPA

Isolates which were stored in deep freezer at -80°C were brought to room temperature for DNA extraction according to the WHO guide line. Briefly, $100\mu\text{l}$ of Lys-A solution, and $50\mu\text{l}$ of very well mixed isolates were added in to 2ml cryotube very carefully and heated at 95°C in (PQLAB) for 5 minutes to kill the bacteria. The tubes were then centrifuged at 13,000 RPM (MIKRO 200) for 5min then $100\mu\text{l}$ of Genolyse A-NB was added and vortexed. Finally $100\mu\text{l}$ supernatant expected to contain DNA was transferred to another sterile tube. The Extracted DNA was mixed with Mix A and Mix B and amplified by mixing $5\mu\text{l}$ of DNA with $45\mu\text{l}$ of Mix solution in the thermo cycler. The amplicon was used for Line probe Assay (LPA).

Then $20\mu\text{l}$ of Denaturation Solution (DEN) and $20\mu\text{l}$ of Amplicon was dispensed in a corner of each of the wells used, $20\mu\text{l}$ of amplified sample was added to the solution, mixed well incubated at room temperature for 5mins. Then pre warmed 1ml of Hybridization Buffer (HYB) chemical which was added to each well followed by immersing labeled DNA-Strip in the mix solution and incubated on TwinCubator for 30 minutes to rotate at 300rpm. The HYB was aspirated completely after 30 min and stringent wash solution (STR) chemical was added and again incubated at 45°C for 15 minutes in TwinCubator, which again aspirated. Working solution of conjugate was added and mixed at room temperature then rinsed two times with rinsing solution and washed by distilled water. Finally substrate working solution was added and mixed on TwinCubator for 5 minutes at room temperature and washed with distilled water. The DNA-Strip was extracted and observed the presence of mutation at “rpoB gene, katG gene and inhA genes which are responsible for anti-

TB drugs Rifampicin and INH in accordance with the control of Amplification [AC] and conjugate [CC] in all of the tests which is the indicator of the perfectness of the procedure.

According to “HAIN Life Science Company produces, *Geno Type MTBDRplus 96 version 2.0*. Thin paper coated with DNA and chemicals are found in box, the DNA’s which are responsible for treating TB are found on the strip which also has procedural control bands (CC) Conjugate control, (AC) amplification control and (TUB) the indicator of the sample run is tuberculosis. And the gens coated are [rpoB controlling band, and rpoB WT1, rpoB WT2, rpoB WT3, rpoB WT4, rpoB WT5, rpoB WT6, rpoB WT7, rpoB WT8, rpoB MUT1, rpoB MUT2A, rpoB MUT2B and rpoB MUT3] genes which are the possible genes for treating TB by the anti-TB drug Rifampicin and the possible mutation that a new TB strain can undergo. [katG controlling band, katGWT, katG MUT1 and katG MUT2] genes which are INH anti-TB drug is act on it and the possible kinds of mutation that a new TB can undergo, and at the bottom [inhA control band, inhA WT1, inhA WT2, inhMUT1, inhMUT2, inhMUT3A and inhMUT3B] genes which are responsible for INH acting sites.

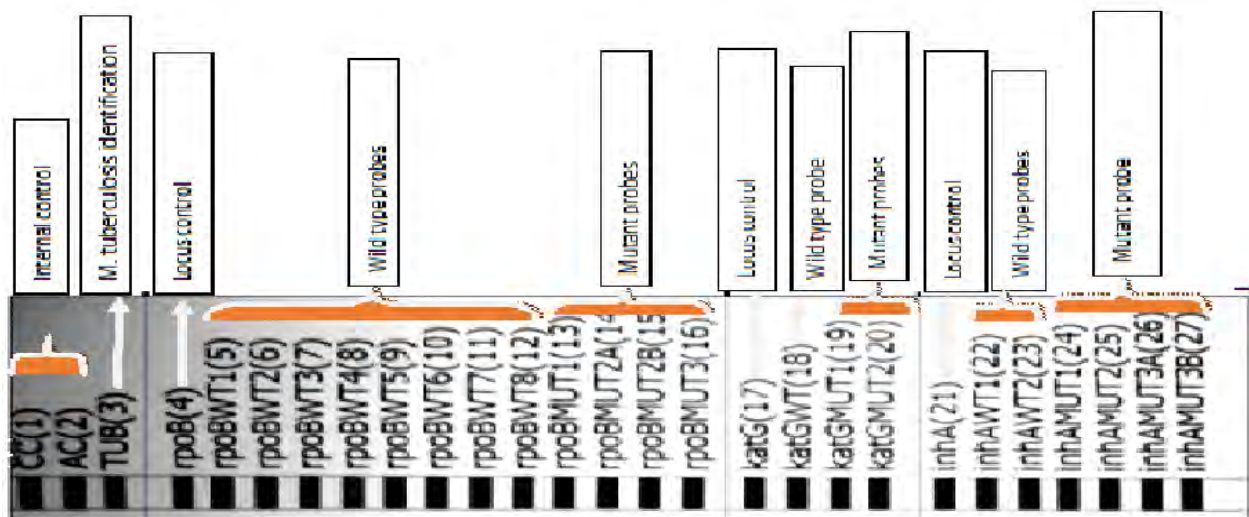


Figure 3:- Description of the 27 reaction zones on the GenoType® MTBDRplus strip

3.8.5. Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, according to the manufacturer's instructions (Kamerbeek *et al.*, 1997). Briefly, the direct-repeat (DR) region was amplified with primers DRa (5'-GGT TTT GGG TCT GAC GAC-30 biotinylated at the 5' end) and DRb (5'-CCG AGA GGG GAC GGA AAC-30). PCR amplification was done for 53 cycles with denaturation, annealing and extension for 1min at 95 °c and 55 °c respectively and extension for 30 sec at 72°c in each cycle. The amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. Sterile water and DNA from *M. tuberculosis* and *M. bovis* was used as a negative and positive control respectively. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The hybridized PCR products were incubated with streptavidin-peroxidase conjugate, and signal detection was obtained with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, England), followed by exposure to X-ray film (Hyperfilm ECL; Amersham) according to the manufacturer's instructions. The X-ray film was developed and washed using standard photochemical procedures.

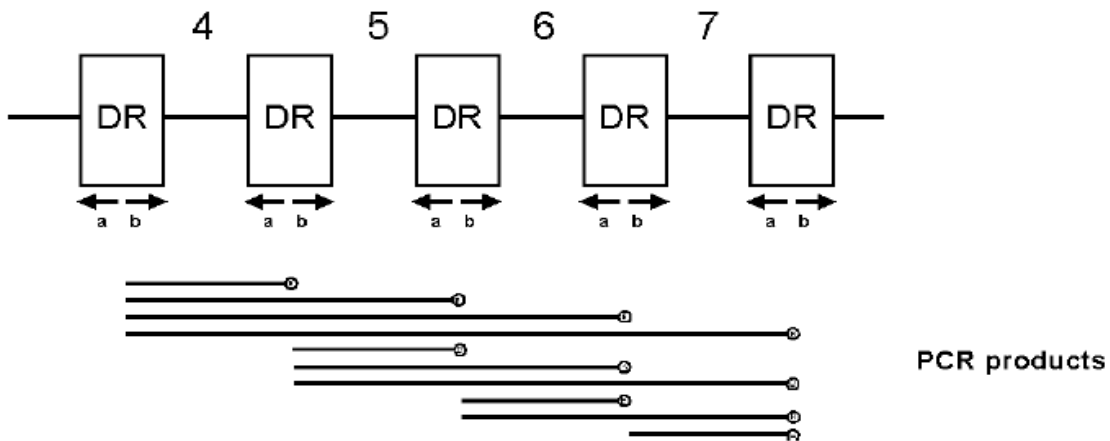


Figure 4:- Principle of DNA amplification of the DR region of *M. tuberculosis* complex bacteria. The two primers, a and b will lead to the amplification of any spacer or a stretch of neighboring spacers and DR's

3.9. Research Communication

The results of our findings were communicated to the concerned body, especially concerning the five patients who became positive for MDR-TB. They were brought to MDR-TB treating center at Adama Hospital in collaboration with Adama Regional Laboratory. All family members of MDR-TB patients were traced and linked to researchers at HIRSCH-INSTITUTE OF TROPICAL MEDICINE who are working on contact tracing of TB patients in Arsi University. Those TB patients which were resistant for only INH were called and asked for their improvement of their treatment and three of them answered they are not cured and agreed to come to Arsi University Asella teaching hospital. When checked by AFB microscopy, they were negative and but GenXpert, positive. Then treated for INH for 9 months and advised to follow their medical status correctly and keep not to infect other family members.

3.10. Ethical Consideration

Ethical approval letter was obtained from Department of Ethical Review Committee (DERC) of Department of Microbiology, Immunology and Parasitology (DMIP), College of Health Science, Addis Ababa University. Support letter was also obtained from Oromia Regional Health Bureau and Arsi zone health Bureau. The purpose and benefit of the study was explained to each study participants by the sample collectors in each study site and those volunteers had been requested to sign consent form and participate the study.

3.11. Data analysis

Socio-demographic and clinical data obtained through questionnaires and the results of laboratory tests were entered into EPI-Info (Version 2004) and Microsoft Excel data record files. Statistical analysis was performed using SPSS software packages (version 21.0 for windows). Tables and graphs were used to present the socio-demographic characteristics of the study subjects after analysis of some selected variables with relevant statistical analysis.

Spoligotype results were analyzed and interpreted based on SpoLDB4 and SITVIT databases. Spoligotype patterns were compared with that found in the SpoLDB4 database and we used SPOLCLUST to assign families/subfamilies for orphan spoligotypes. Lineage was assigned on the

basis of spoligotype Large Sequence Polymorphisms (LSPs). Spoligotype rules for each lineage was based on the presence or absence of specific spacer sequences in the direct repeat locus (i.e., absence of 1–34 spacers for East-Asian; absence 33–36 for Euro-American; absence of 29–32, 34 spacers, and presence of 33 spacer for Indo-Oceanic; and absence of 4–7 and 23–34 spacers for East-African Indian) (Gagneux and Small, 2007).

3.12. Data quality assurance

The quality of data was controlled starting from the time of questionnaires development. The questionnaire was developed by reviewing relevant literatures on the subject to ensure reliability and developed in English. The questionnaire which was prepared in English was translated into Afan Oromo and Amharic. Onsite training was given for sample and data collectors on the purpose and the aim of study. Concerning the reagents and chemicals that used for staining ZN-AFB and LED-Florescent, known positive and negative control had been run daily as a control. During culture test the quality was monitored by running sample free LJ and running known H-37 TB strain and the working environment was disinfected by 1% bleach (mix of 20ml of 5% bleach and 80ml of distilled water). During PCR, the area of PCR mix has been cleaned with “DNA away” before and after each mix. We used a separate mix room and amplified the mix in a different room to prevent cross contamination.

Chapter Four

Result

4.1. Socio-demographic background of the Study participants

A total of 150 smear positive pulmonary TB patients were considered in this study. The participant's age range was from 6 -72 years with the mean (\pm SD) of 29 (\pm 13.25). More than half 79 (52.7%) were married and 60 (40.0%) of them were females. Sixty six (44%) of the study participants had knowledge about tuberculosis, 23 (15.3 %) and 4 (2.7%) of the study participants had history of hospital admission and imprisonment, respectively and 34 (22.7%) of the patients had history of contact with tuberculosis patients in their home or nearby friends. Likewise 58 (38.7%) of the study participants had having a habit of drinking raw milk.

Table 3:- Table shows Socio-demographic characteristic of study participants

Variables	Variable sub-class	Unit frequency	Percentage (%)
Age	<15	15	10
	16-25	66	44
	26-35	33	22
	36-45	18	12
	46-55	11	7
	>55	7	5
Sex	Male	90	60.0
	Female	60	40.0
Educational level	Illiterate	41	27.3
	Elementary	58	38.7
	High school	32	21.3
	College and above	19	12.7

Marital status	Single	68	45.3
	Married	79	52.7
	Separated	3	2.0
Occupation	Unemployed	14	9.3
	Civil servant	19	12.7
	Student	43	28.7
	Daily laborer	7	4.7
	Merchant	53	35.3
	Farmer	13	8.7
	Other	1	0.7
HIV Status	Positive	2	1.3
	Negative	88	58.7
	Not screened	60	40.0
Pregnancy	Yes	1	1.7
	No	59	98.3
Habits of drinking alcohol	Some times	2	1.3
	Always	1	0.7
	Not at all	147	98.0

4.2. Smear microscopy result

During the study period in Arsi zone including seven woredas having one governmental Hospital which diagnose Tuberculosis by both microscopic methods Florescence and Zehilnelson, one private Hospital, one private higher clinic and eight governmental Health center which all uses only Zehilnelson diagnostic method, were participated for this study, totally 150 smear confirmed TB positive sputum's were collected. Of 150 smear observed, 92/150 (61.3%) were graded +1, 27/150 (18%) were graded +3, 11/150 (7.3%) were graded +2 and 20/150 (13.3%) were graded scanty.

4.3. Molecular characterization of Mycobacterium tuberculosis in Arsi Oromia Ethiopia

4.3.1. Genetic diversity and family assigning






Characterization of the strains based on Bio-numeric (1&0) produced from spoligotyping in to Lineage, Family and strain was done based on SpolDB4 (Spoligotyping international data base 4) for (up to SIT-1938) and SITVIT web, [http:// www.pasteur-guadeloupe.fr:8081/SITVIT released in 2012](http://www.pasteur-guadeloupe.fr:8081/SITVIT_released_in_2012). Those strains which didn't find in either of the two were designated as UK (Unknown).

Totally 65 isolates had registered SIT on SpolDB4, specifically the SIT number found in this study was, 35 of 130 had an SIT NO. 53, 5 of 130 had an SIT NO 149, 5 of 130 had an SIT NO 118, and SIT NO (37, 120, 777, and 1547) was found two times each and SIT NO (26, 50, 117, 142, 185, 247, 699, 736, 952, 1129, 1318 and 1821) was found one times each and the remaining 65 of 130 had no SIT NO on SpolDB4. In this classification, SIT NO 26 and 247 was assigned as CAS1 DELHI strain type.

4.3.2. Cluster classification

Cluster classification (isolates which have similar missing and existing of 43 genes on spoligotyping) showed that, five clusters was found. Thus, 34 isolates were missed genes only from 33 up to 36, five isolates were missed genes from 10 up to 20 and from 33 up to 36, six isolates were missed gene number 15, 20 and from 33 up to 36, five isolates missed gene number 15 and from 33 up to 36 and two isolates missed gene number 20 and genes from 33 up to 36. The rest of isolates has no similar point of missing and expressing indicating that those which have similarity are in expected to be acquired the disease from the point of index even though which patient had the disease earlier and who was latter, and for those which have no common points of missing genes are expected to acquire from different origins or the bacteria by itself may get loss or express genes.

Table 5:- Distribution of clusters of Tuberculosis isolates found in Arsi zone Oromia region Ethiopia.

Number of isolates found (N=130)	Missed regions on Spoligotyping	Spoligotyping description	SIT Number on SpolDB4	Strain type
32/130	Only from 33 up to 36		53	T1
5/130	Region 15 and from 33 up to 36		118	T2
2/130	Region 20 and from 33 up to 36		120	T1
6/130	Region 15, 20, and from 33 up to 36		NA	NA
5/130	Region from 10 up to 20 and from 33 up to 36		NA	NA

4.3.3. Lineage and family classification

Of the 130 spoligotyped isolates, 104 (80.0%) belongs to Euro-American lineage (lineage-4), 15 (11.5%) Indo-Oceanic lineage (lineage-1), 9 (6.9%) Unknown lineage, 1(0.8%) West African-1(lineage-5) and 1(0.8%) *Mycobacterium bovis*. From the total 104 Euro-American lineage 46 (44.2%) belongs to T family, 21(20.2%) T3 family, 18 (17.3%) T3-ETH family, 5(4.8%) H3-Ural-1, 4(3.8%) Manu 2, 3 (2.9%) T1-RUS1, 2(1.9) X1, and 5(4.8%) X2, H1, EAI, T2 and T1-RUS2. The identified 15 Indo-Oceanic lineages were also further classified in to families where, 5 (33.3%) belongs to Manu2 family, 4 (26.7%) CAS1-Delhi family, 2 (13.3%) AFRI family, 1 (6.7%) H3 family, 1(6.7%) ‘T’ family, 1(6.7%) Canetti and 1(6.7%) EA14-VNM family.

Table 6:- Lineage and family classification of isolates collected from Arsi zone Oromia region Ethiopia

Complex	Major lineage	Strain type
MTC	Euro American	EAI
		T1-RUS1
		T
		X
		T3-ETH
		H3
		T
		T3
		X1
		H1
		H3-Ural-1
		Manu-2
		T1-RUS2
	Indo-Oceanic	T
		CAS-1
		Manu-2
		AFRI
		EA14-VNM
		Canetti
	Un Known	H3
		CAS1-Delhi
		CAS-1
	M.bovis	Beijing
West Africa-1	PINI2	

4.3.5. Distribution of strains in different Woreda

The distribution of the identified strains (families) at Woreda level from which the samples were collected is shown in table 9. The strain type “T” was the dominant strain (n=) circulating in the area and found in all study woreda’s at higher percent, followed by “T3-ETH” family. Tiyo woreda was the host for majority of the strain types identified

Table 9:- Distribution of MTC family in Woreda’s

KBBN (Family)	Family * Woreda Cross tabulation							Total
	% within Woreda N=130							
	Woreda’s							
	Tiyo	Hetosa	L.Hetosa	Shirka	Munessa	L. bilbillo	Robe	
T	30.8%	15.4%	50.0%	53.8%	40.0%	47.6%	30.8%	36.2%
T3-ETH	11.5%	7.7%	25.0%	15.4%	10.0%	14.3%	23.1%	13.8%
X1	1.9%			7.7%				1.5%
H3-Ural-1		7.7%		7.7%	10.0%		15.4%	3.8%
T3	21.2%	30.8%	12.5%	7.7%		19.0%		16.2%
CAS1-Delhi	3.8%	23.1%	12.5%			14.3%	15.4%	8.5%
Manu2	9.6%				10.0%			4.6%
CAS1	3.8%							1.5%
T1-RUS1	3.8%						7.7%	2.3%
M. Caprae							7.7%	0.8%
H1				7.7%				0.8%
AFRI	1.9%				10.0%			1.5%
Beijing		7.7%						0.8%
H3	7.7%							3.1%
EAI					10.0%			0.8%
X2	1.9%							0.8%
T2		7.7%						0.8%
EA14-VNM	1.9%							0.8%
T1-RUS2					10.0%			0.8%
Canetti						4.8%		0.8%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

4.4. Drug susceptibility testing (DST)

The cumulative resistance in the study area was 10/130 (7.7%). (Table 11). Of 130 isolates Rifampicin and Isoniazid mono resistance were detected in 2 (1.5%) and 5 (3.8%) of isolates. Multidrug resistance (i.e. those isolates which were resistant to both rifampicin and isoniazid) was detected in 3 (2.3%) out of the 130 isolates.

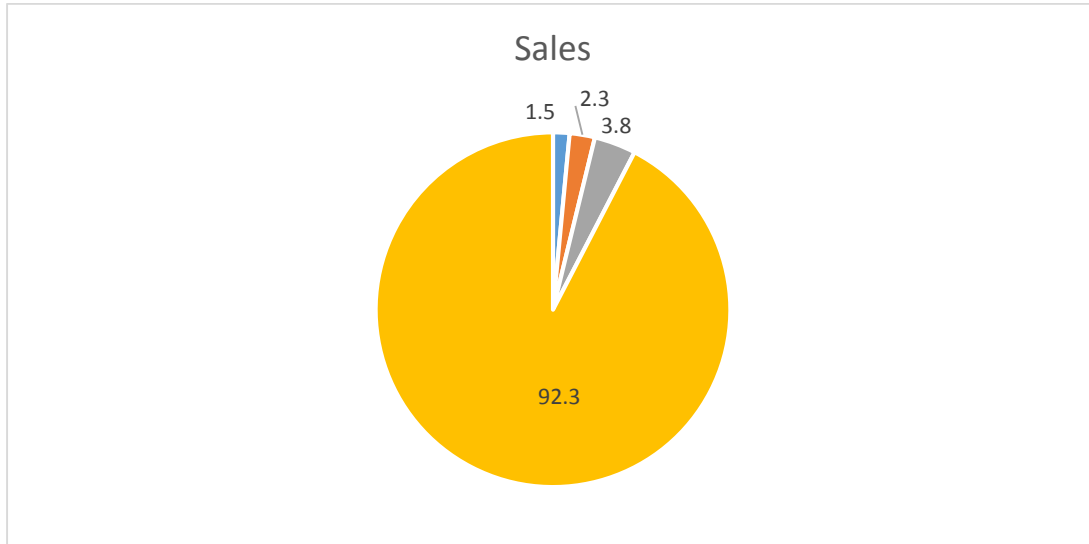


Figure 5 :- Drug susceptibility distribution of TB positive samples collected from study participants from February 2015 to June, 2015 in Arsi Oromia Ethiopia.

On the other hand, two patient samples on LPA were deleted for the gene *rpoB* WT7 and *rpoB* WT8 but didn't express any of the mutant gene (*rpoB* MUT1, *rpoB* MUT2A, *rpoB* MUT2B and *rpoB* MUT3).

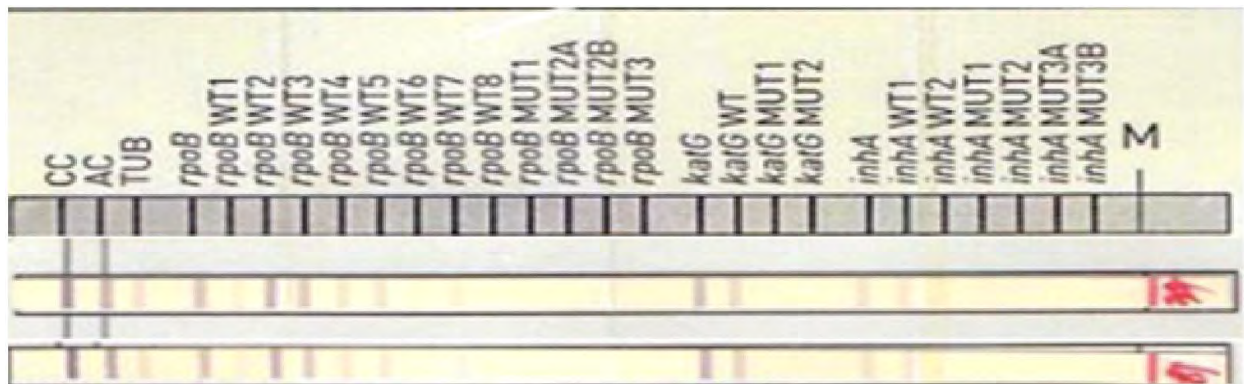


Figure 6:- LPA of missing WT and MUT gene

Chapter Five

Discussion

In this study we have described the molecular epidemiology and drug susceptibility of sputum samples which were confirmed smear positive and new tuberculosis patients which were collected from seven woreda's of Arsi, Oromia region, Ethiopia.

For this study we collected and cultured 150 samples from seven study area, of those 130 of them were grown and done for molecular characterization and drug sensitivity testing, the rest 20 samples were fail to grow on solid Lowisten Jenson media, when I trace back the possible reason for not grow was, majority 14 of 20 samples were having low burden of Tuberculosis bacillus which were graded scanty and +1 in the field, and the rest 6 of 20 were bloody sputum which have had seen growth covered by fungal species.

Of the study participants 44% were in the age range of 16-25, which is the productive age group, and in line with many other researches including WHO 2015 report. Males were also seen dominating females in this study, accounting 60% (90/150), which is similar like other researches done by Berhe *et al.*, 2012; Ejeta *et al.*, 2014. On the other hand 58 (38.7%) of the study participants were have had a habit of drinking raw milk and one patients which had *M.bovis* is also from this percent.

The molecular characterization on spoligotyping to lineage showed that, 104/130 (80.0%) were Euro-American, 15/130 (11.5%) Indo-Oceanic, 9/130 (6.9%) Unknown lineage, 1(0.8%) West African-1 and 1(0.8%) *Mycobacterium bovis* were identified from SpolDB4 (n=1938) and SITVIT 2012 which indicating the lineage "Euro-American" is more circulating in the study area, thus spoligotyping pattern lineage finding is in line with the finding of Firdessa *et al.* (2013), and Tessema *et al.* (2013) from Ethiopia and Globally the lineage "Euro-American" is the dominating one according to Brosch *et al.* (2002).

From Lineage to strain characterization done for this study showed that, strain type 'T' which accounted 36.2% from this study is the most dominating and found in all seven woredas of the study site with 30.8%, 15.4%, 50.0%, 53.8%, 40.0%, 47.6%, and 30.8% in Tiyo, Hetosa, L. Hetosa, Shirka, Munessa, L. bilbillo, and Robe woredas respectively, followed by T3-ETH type

which accounted 13.8% , indicating that there is a recent transmission of Tuberculosis among the society which I couldn't find and associate with other research done in this country. But it is the dominating strain circulating among all of the seven study woredas thus it could be the answer for the questions why the seven woredas gets high burden of Tuberculosis among the rest 25.

Cluster classification (isolates which have similar missing and existing of 43 genes on spoligotyping) showed that, five clusters was found in which all the study woredas showed they have had a cluster, implies recent transmission like the report of Tostmann *et al.*, 2008; Wirth *et al.*, 2008, Dheda *et al.*, and 2010. The higher rate of PTB and clustering is indicative of defects of the TB control program Aranaz *et al.*, 2003, as seen in Arsi.

The DST pattern shows 3 (2.3%) were resistance for both Rifampicin and INH, 5 (3.8%) were mono resistance for INH and 2 (1.5%) were mono resistance for Rifampicin, so that according to the guide line 5 (3.8%) of the new patients were MDR-TB, in which it has a slight increment from the WHO GTR 2015 estimate which is (3.5%). It has also shown increment from the research done in Eastern Amhara region by (Esmael *et al.*, 2014) which is (1.8%), and also this result has shown increment with other studies like in Addis Ababa and Harar by (Wolde *et al.*, 1986) which was 2% and 1.3% respectively. The two patients which were resistance for Rifampicin was 2/46 (4.3%) from "T" family, INH mono resistant were 2/18 (11.1%) from "T3-ETH", 2/21 (9.5%) from "T3" and 1/11 (9.1%) from CAS1-Delhi.

When we see the socio characteristics of those patients which were mono resistant for INH has no previous exposure for the drug as prophylaxis, but have had a contact with Tuberculosis infected patients, and those who were MDR (resistant for both INH and Rifampicin) are females and single and their age was between 16-20 and the type of the strain specifically was CAS1-Delhi, T3-ETH and T, and all of them were from different woreda's, has no exposure with TB patient in family and nearby friend and no hospital admission history and also negative for HIV

On the other hand two patient samples on LPA were deleted for the gene *rpoB* WT7 and *rpoB* WT8 but didn't express any of the mutant gene (*rpoB* MUT1, *rpoB* MUT2A, *rpoB* MUT2B and *rpoB* MUT3) which needs further diagnosis by another molecular technique. We did GenXpert to detect for the Rifampicin resistance and both of them were detected no Rifampicin resistance gene.

But according to the guide line seated by FIND (foundation for innovation new diagnostics) 2012, the absence of a signal for at least one of the rpoB WT probes with or without the presence of a MUT band indicates that the strain should be predicted to be resistant to RMP. The presence of less common mutations within rpoB that result in absent bands for either of WT reaction zones (which lack corresponding mutation specific capture probes) also should be interpreted as resistance to RMP.

Biochemical, Immunological and molecular characterization of *M. tuberculosis* has led to the identification of several antigens which may be useful in the development of improved diagnostic methods in order to discriminate between *M. tuberculosis* complex and other than *M. tuberculosis* (MOTT). *M. tuberculosis* has known to secrete more than 33 different proteins, one of the predominant protein is MPT64 was found in the culture fluid of only strains of M tuberculosis complex. That is why rapid SD cassette chromatographic testing Capilia device was developed to differentiate between non tuberculosis complex members (NTM) from the complex, and 130 samples was performed for capilia cassette tests and 12/130 (9.23%) of them becoming negative which is interpreted as they are not the member of the complex even though the type of the strain specifically is not distinguished. But on spoligotyping they found MTC specifically they were under lineage of Euro-American and Indo-Oceanic.

Limitation of the study

The limitation of this study is that, the numbers of isolates are smaller and study did not included all woreda's in Arsi zone to provide a true picture of possible types of *M. tuberculosis* lineages, specific strain and prevalence of MDR TB.

Conclusion and Recommendation

Conclusion

In conclusion, our study confirms a highly diverse population structure of *M. tuberculosis*, the presence of phylogenetic lineages that were not described before and a predominance of the Euro-America lineage in the Oromia region, Southwest Ethiopia. Our study also showed new isolates which were resistant to anti-TB drugs including multidrug reissuance. The high rate of recent transmission underlines active transmission of *M. tuberculosis* including drug-resistant strains, and consequently the inefficacy of TB control program in the study area. This emphasizes the importance of strengthening laboratory diagnosis of TB including culture and drug susceptibility testing, intensified case finding and treatment of TB patients according to the ongoing DOTS program to interrupt the chain of transmission within the community.

Recommendations

1. MDR-TB treating centers should have to be established at zonal level in Arsi for controlling.
2. More research has to be done especially on those re-treated TB patients to know the prevalence of MDR-TB, hence the prevalence from new patients are showing some increments.
3. Arsi University which is found in Asella, the town of the Zone should have to give health education at sub-kebele level and should have to promote such researches to be done, and the University Teaching Hospital in collaboration with Oromia and Federal Ministry of health should have to establish TB culture laboratory.
4. As the molecular pattern showed, the lineage Euro-American is more dominantly circulating typically strain type “T” which is the indication of there is a very rapid cross exchange of TB infection among the society, so if proper health education and intervention was given the disease can be stopped, so the concerned organizations should take their responsibility to stop the spread.

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Löwenstein-Jensen (LJ) medium

Löwenstein-Jensen (LJ) medium is most widely used for tuberculosis culture. LJ medium containing glycerol favours the growth of *M. tuberculosis* while LJ medium without glycerol but containing pyruvate encourages the growth of *M. bovis*.

Ingredients

1. Mineral salt solution

- ✓ Potassium dihydrogen phosphate anhydrous (KH₂PO₄)2.4g
- ✓ Magnesium sulphate (MgSO₄. 7H₂O)0.24g
- ✓ Magnesium citrate0.6g
- ✓ Asparagine..... 3.6g
- ✓ Glycerol (reagent grade)12ml
- ✓ Distilled water..... 600ml

121°C for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator for longer.

2. Malachite green solution 2%

- ✓ Malachite green dye2.0g
- ✓ Sterile distilled water100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store.

3. Homogenized whole eggs

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap for 30 minutes and Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of complete medium

- Mineral salt solution600ml
- Malachite green solution20ml
- Homogenized eggs (20-25 eggs, depending on size)1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes, and the tops are securely fastened then inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Annex 1 Informed Consent Form

Identification No -----, lab No -----

I read /well informed about the nature of the study entitled “

Molecular epidemiology and drug sensitivity of mycobacterium tuberculosis isolates among new pulmonary tuberculosis patients in Arsi, Oromiya, Ethiopia” and I have understood that no pain and no effect during the procedure and only leftover sample is to be used. And also, she/he asked my willingness to allow the leftover sample to be collected for the research purpose. Finally, she/he told me that this will be certain if I agree on the following points and signed bellow.

- ✚ I understood the objective of the study
- ✚ I understood that the sample won't be used for farther study, and after completion of the whole procedure, the leftover sample will be discarded safely
- ✚ I am aware of any information describing my identity, collected using questionnaire and, won't be disclosed
- ✚ I understood report of my result won't include my name and my specific identity.
- ✚ I understood that I won't get money for being part of the study except compensation for transportation and elapsed time if only the result become positive.
- ✚ I clearly informed as I have the right to refuse to participate and withdraw if I change my mind from the study at any time
- ✚ I understood that my refusal to take part in this study won't have impact to the normal diagnosis and to my future medical follow up.

I have had enough time to think over it freely and I understood it well in my language. I found it would have positive impact in the investigation of my case. My agreement to this consent is without any external enforcement, and will be confirmed by my signature, below.

The information sheet was given/ explained to me by: -----, signature -----

Name of participant: -----, signature -----, phone -----

Individuals who were around 1. Name _____ Sign _____

Annex 2 Declaration Sheet

Title of the study: - The objectives of this study is to characterize the diversity of M.tb strains circulating, determine the drug susceptibility of the isolates among new PTB+ patients in Arsi zone Oromiya region Ethiopia

Prioritize the patient's safety and I am responsible for the role(s) I am going to play and showing by my signature in the table below.

S. No.	Investigators	Responsibilities	Signatures	Remark
1	Fekadu Tamrat	Write up Material supply Sample care and transportation Processing the whole laboratory activity principally.		
2	Dr. Tamrat Abebe	Supervising the whole process.		
3	Dr. Adane Mihret	Supervising the laboratory activity part and Write up Material supply		
4	Dr. Ketema Tafesse	Supervising the laboratory activity part and Write up Material supply		
5	Prof. Gobena Ameni	Supervising the laboratory activity part and Write up Material supply		

Annex 3 Questionnaire

ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES, DEPARTMENT OF
MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

Questionnaire format for the assessment of the common risk factors of Tuberculosis

Date of Interview _____ PIN _____ Laboratory Code No _____

Part I. Addressees

1. Region _____ 2. Zone _____
3. Woreda _____ 4. City _____
5. Kebele _____ 6. Gote (Sub-kebele) _____
7. House No _____ 8. Phone No _____

Part II .Socio-Demographic Information

9. What is Your Age? _____ (years)
10. Sex? 1. Male 2. Female
11. Religion? 1. Orthodox 2. Muslim 3. Protestant 4. Catholic 5. Other _____
12. Educational Level? 1. Illiterate 2. Elementary 3. High School
4. College and Above
13. Marital Status? 1. Single 2. Married 3. Separate 4. Widowed 5. Divorced
14. Number of Family Members? 1. ≤ 2 2. 3 – 5 3. 6 – 8 4. 9 – 11 5. ≥ 12
15. Occupation? 1. Unemployed 2. Civil Servant 3. Student 4. House Wife
5. Daily Laborer 6. Pensioned 7. Merchant 8. Farmer
9. Driver 10. Other _____

Part III. Predisposition for TB

16. Do you know about TB? How it is transmitted from one another? 1. Yes 2. No
17. If yes, are you practicing others not to contract this disease by covering your mouth during talking and coughing? 1. Yes 2. No
18. Is there someone who is TB patient in your household? 1. Yes 2. No 3. I don't know
19. Have you ever had a friend, neighbor, or school mate with TB? 1. Yes 2. No

3. I don't know

20. Habit of Drinking Milk 1.Raw 2.boiled 3.both (boiled and raw)

4. I do not drink

21. Do you have the habit of eating raw meat 1.Yes 2. No

22. Do you have history of Hospital Admission? 1. Yes 2.No

23. Do you have previous history of imprisonment? 1. Yes 2. No

24. Do you Drink Alcohol? 1. Sometimes drink alcohol 2. Always drink Alcohol

3. I didn't drink Alcohol

25. Do you know your HIV test result? 1. Yes 2. No

If yes, what was your HIV Test Result 1.Positive 2.Negative 3. On ART

26. Are you Pregnant (For Female) 1.Yes 2. No

If yes gestational period in weeks _____?

27. Do you Smoke Cigarettes? 1. Sometimes I smoke cigarettes

2. Always I smoke cigarettes 3. I didn't Smoke cigarettes

28. Do you know someone who is treated for TB more than one times? 1. Yes 2.No

29. If yes what relationship do you have with that person? 1. Family member 2. Close friend

3. Noncontact person

30. Do you have contact with MDR-TB Patients? 1. Yes 2.No

31. Previous history of TB treatment? 1. Yes 2. No [New]

32. If yes, did you completed the treatment? 1. Relapse 2. Default 3.Failure

33. Contact person: - Name _____

Relationship 1. Mother 2. Father 3. Children 4. Brother/Sister 5. Other _____

Telephone of contact person _____

Name and Signature of the Data Collector/Interviewer _____

Annex 4:- Maps

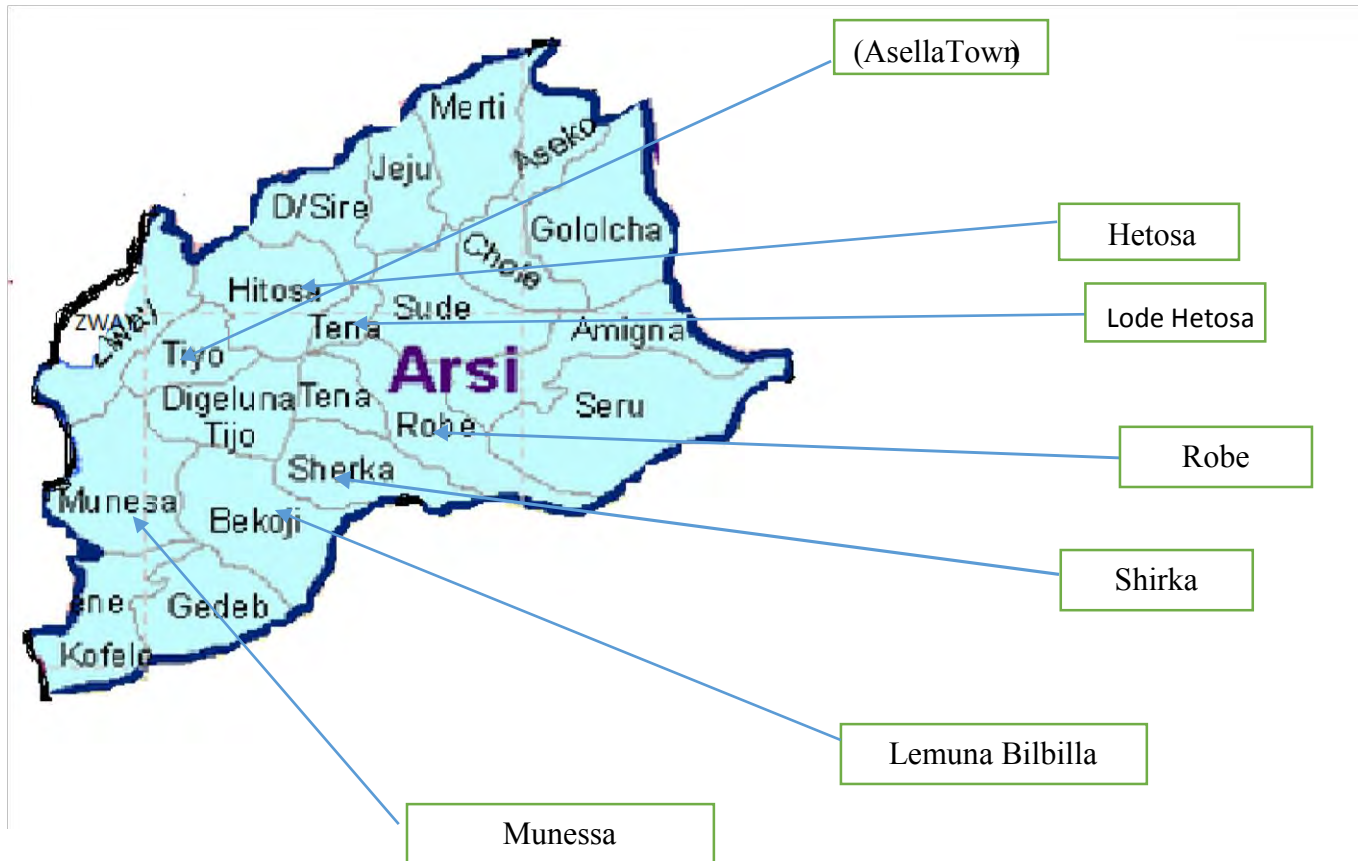


Figure 7:- Maps