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Epidemiology and Drug Resistance Pattern of *Mycobacterial* Isolates among HIV positive and HIV negative TB patients using Conventional and Molecular Methods in South east Ethiopia

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LIST OF ABBREVIATIONS

ACP	Acyl Carrier Protein
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immune Deficiency Syndrome
ALERT	All Africa Leprosy and Tuberculosis Research, Rehabilitation and Training Center
ART	Anti-Retroviral Treatment
ARV	Antiretroviral
ATCC	American Type Culture Collection
BMI	Body Mass Index
Bp	Base pair
CDCT	Communicable Disease Control Team
CTAB	Cetyl-Trimethyl-Ammonium Bromide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOTS	Directly Observed Treatment Short course
DR/DVR	Direct Repeat/ Direct Variable Repeat
E/ EMB	Ethambutol
EHNRI	Ethiopian Health and Nutrition Research Institute
FASI	Fatty Acid Synthase I
FDC	Fixed Dose Combination
H/INH	Isoniazid
IS	Insertion Sequence
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Lowenstein Jensen
MCF	McFarland
MIC	Minimal Inhibitory Concentration
MDR-TB	Multidrug-resistance Tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
MoH	Ministry of Health

mRNA	Messenger Ribonucleic acid
NAD	Nicotine Adenine Dinucleotide
OADC	Oleic Acid Albumin Dextrose Catalase
OD	Optical density
OPD	Outpatient Department
PBS	Phosphate Buffered saline
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
QA	Quality Assurance
QC	Quality Control
RFLP	Restriction Fragment Length Polymorphisms
RMP/R	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic acid
S/STM	Streptomycin
SDS	Sodium dodecyl sulfate
SNNPR	Southern Nations and Nationalities and People's Region State
TB/HIV	The intersecting epidemics of TB and HIV Co-infection
TCH	Thiophene-2-Carboxylic acid Hydrazide
TLCT	TB and Leprosy Control Team
VCT	Voluntary Counseling and Testing
WHO	World Health Organization

GLOSSARY

Amplification of drug resistance: A phenomenon where additional drug resistance is acquired by an already drug resistant isolate.

Any type drug resistance: Single, double, triple, quadruple or resistance to five drugs.

Combined resistance: Resistance to more than one drug (synonymous with multiple drug resistance).

Case of tuberculosis: A patient in whom tuberculosis has been confirmed by bacteriology or diagnosed by a clinician.

Category I TB patients New smear positive TB patients and severely ill smear negative and extra-pulmonary TB.

Category II TB patients Return after default, treatment failure and relapse after treatment completed or cure.

Category III TB patients Smear negative and extra-pulmonary TB patients who are not seriously ill.

Chronic cases A patient who is still smear positive at the completion of re-treatment regimen.

Cohort A group of patients in whom TB has been diagnosed and who were registered for treatment during a specified time period.

Cured An initially smear-positive patient who was smear-negative in the last month of treatment and on at least one previous occasion.

Defaulters A patient who has been on treatment for at least 4 weeks and whose treatment was interrupted for more than 8 consecutive weeks.

DOTS-plus: TB treatment programme where DOTS is supported by the use of second line anti-TB drugs.

Drug resistance without previous treatment for TB: Drug resistance detected in individuals not previously treated with anti-TB drugs or treated for less than a month.

Drug resistance with previous treatment: Drug resistance detected in individuals

previously treated with anti-TB drugs for more than a month.

Mono-resistance: Resistance to a single drug.

Multidrug-resistance (MDR): Resistance to at least Rifampicin and Isoniazid.

Multiple drug resistance: Resistance to more than one drug including Multidrug resistance.

New case A patient who has never had treatment for tuberculosis or who has taken anti-TB drugs for less than 1 month.

Phenotypic lag: The delay in expression of newly arisen mutation for significant time after it arises, such as one or more cell generations.

Relapse: A patient who has been declared cured or treatment completed of any form of TB in the past, but who reports back to the health service and is found to be smear–positive or culture positive.

Re-treatment case: A patient previously treated for tuberculosis, undergoing treatment for a new episode of bacteriologically-positive tuberculosis (expressed as a percentage of the number registered in the cohort).

2S(E) RHZ/6HE: A DOTS strategy for category-I TB patients where Streptomycin or Ethambutol, Rifampicin, Isoniazid and Pyrazinamide are taken for two months followed by 6 months of Isoniazid and Ethambutol.

Smear-positive pulmonary: A patient with at least 2 initial sputum smear examinations AFB+; or one sputum examination AFB+ and radiographic abnormalities consistent with active Pulmonary tuberculosis as determined by a clinician; or one sputum specimen AFB+ and culture positive for *M. tuberculosis*.

Treatment failure: A patient who, while on treatment, remained smear-positive or became smear-positive at the end of five 'months' or later, after commencing treatment.

TB case definition: An individual starting a full course of anti-TB treatment based on clinical and/or radiological evidence.

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ABSTRACT

Background:- Tuberculosis is the leading cause of death in hospitalized patients. It has not yet been investigated whether MDR-TB contributes to the reported high mortality. There has also been no study on the molecular epidemiology of TB and M. tuberculosis strain types have not yet been characterized in the area. Nationwide, there have been no previous reports on drug resistance in individuals treated with 4 fixed dose combination (FDC) anti-TB drugs. Shashemene is one of the known locations for high prevalence of TB and HIV in Ethiopia. The TB-HIV co-infection rate in the age group of 15-49 years was estimated to be 47% in the area (MoH, 2004).

Objectives:-To determine the prevalence of resistance to the four first line anti-TB drugs with and without HIV co-infected TB patients and to investigate association of TB bacteria strain types with drug resistance.

Methods: - A cross-sectional survey on anti-TB drug resistance was conducted in four health centres and one Hospital in and around Shashemene in Ethiopia. Sputum and blood samples were collected from all consenting smear positive pulmonary TB patients visiting the sites between May 2006 and December 2006. Sputum was digested and decontaminated using Petroff's method with 4% NaOH and cultured on Lowenstein Jensen media. Drug sensitivity tests were performed on isolates using the proportion method on Middlebrook 7H10 media and 10% OADC enrichment. Pateints were screened for HIV with rapid assays (Determine®, Capillus® and Unigold®) according to national guidelines. Species were identified with biochemical (Thiophene-2-Carboxylic acid Hydrazide [TCH] test) and DNA based methods (species specific PCR amplification with RD4 primer). Drug resitance was further characterized using PCR based mutation analysis as a rapid and simple technique for diagnosis of drug resistance in tuberculosis. We described a simple multiplex allele-specific (MAS)-PCR assay to detect mutations in the second base of the katG gene codon 315, including AGC →ACC and ACA (Ser→Thr) substitutions that confer resistance to isoniazid (INH) in M. tuberculosis clinical isolates. We used PCR-RFLP assay for rpsL43 mutation analysis, in the absence of mutation in AAG→AGG position in MboII digest rpsL43 and the fragment was a 210-bp and 60-bp. If a mutation in codon 43 resulted

in a 270-bp fragment.

***Results and Discussion:* -** A total of 292 (264 new and 28 previously treated) patients were included in this study. Out of these, 82.5% (241/264) were culture positive. Sensitivity test results were available for 217 isolates from new cases and 24 isolates from previously treated patients. Among the isolates, 80% (174/217) of those from new patients and 54.2% (13/24) of those from previously treated patients were sensitive to all drugs tested. Prevalence of MDR-TB among new cases was 0.9% (2 isolates). Resistance to INH, RMP, STM and EMB was 12.9% (28/217), 1.8% (4/217), 16.6% (36/217) and 4.1% (9/217) respectively. In previously treated patients INH, RMP, STM and EMB resistance was 37.5% (9/24), 8.3% (2/24), 41.7% (10/24) and 12.5% (3/24) respectively. There was also no association observed between drug resistance among new cases and HIV. The high prevalence of the *kat*G315 ACC mutant allele among INH resistant and *rps*L43 AGG mutant allele among STM resistant *M. tuberculosis* clinical isolates in the Shashemene area, Southeast Ethiopia, 83.8% (31/37) and 55.56% (20/36) for all isolates was observed.

***Conclusion*:** Prevalence of Multi-drug resistant TB in the Shashemene area is comparable with the national report. *M. bovis* was not isolated from pulmonary TB in the area.

Key words: Proportion methods; Multi-drug resistant tuberculosis; Tuberculosis; Human Immunodeficiency Virus; Analysis of TB drug resistant mutants;

1. INTRODUCTION

1.1 Mycobacterium tuberculosis Complex

Mycobacterium tuberculosis is a fastidious, slow growing, lipid-rich, hydrophobic and acid fast bacterial rod shape which resists decolorization with acid alcohol. It has no outer membrane, rather has a cell wall made up of different macromolecules, namely peptidaglycans, arabinogalactan, mycolic acid and lipopolysaccharide or lipoarabinomana (LAM) which is anchored plasma membrane. Mycolic acid is the major component of the cell wall envelope, greater than 50% by weight and defines the genius mycobacteriae. The staining characteristic of *M. tuberculosis* is due to the mycolic acid which resists decolorization by acid alcohol.

Mycobacterium tuberculosis is complex bacteria which causes an infectious disease tuberculosis. It includes *M. tuberculosis, M. bovis, M. africanum* and the recent additions of *M. canetti* and *M. microti*, which are genetically closely related sub-species. Repetitive DNA elements such as insertion sequence (*IS6110)* and direct repeat have been found to be restricted to the *M. tuberculosis* complex (van Soolingen *et al*., 1997). The complete genome of the *Mycobacterial*, strain H_{37}Rv has been sequenced and is known to contain 4,411,529 bps consisting of about 4,000 genes with Guanine and Cytosine (G+C) content of 65.6% (van Soolingen *et al*., 1997).

The natural reservoir of *M. tuberculosis* and *M. africanum* is limited to humans while that of *M. microtti* is rodents and *M. bovis* is a wide range of wild, domestic animals and occasionally humans.

1.1.1. Diagnosis of Tuberculosis

The conventional diagnosis of TB requires detection of acid-fast bacilli in sputum via the Ziehl-Neelsen straining method. The organisms must then be cultured from sputum. First, the sputum sample is decontaminated with 4% NaOH. This kills other contaminating bacteria but does not kill the *M. tuberculosis* present because *M. tuberculosis* is resistant to alkaline compounds by virtue of its lipid layer (Kenneth, 2005).

Two media used to grow *M. tuberculosis*, which are an agar based Middlebrook and an egg based Lowenstein Jensen (LJ) media. *M. tuberculosis* colonies are small and buff colored when grown on either medium. However, this method of culturing can take 3-8 weeks to yield visible colonies. As a result, another method is commonly used called the BACTEC System. The media used in the BACTEC system contains radio-labeled palmitate as the sole carbon source. It multiplies, breaks down the palmitate and liberates radio-labeled CO_2 which can indicate the presence of bacteria multiplication in the BACTEC system. Its growth can be detected in 9-16 days whereas 3-8 weeks using conventional LJ media (Kenneth, 2005).

1.2. Global/National Epidemiology of TB

Tuberculosis (TB) is one of the leading cause of death in the world. The disease affects 2 billion people which is equal to one-third of the entire world population. Globally around 9 million people develop tuberculosis and 2-3 million people die every year (Kenneth, 2005). This increased incidence occurs mostly in Africa and Asia, where the highest prevalence of co-infection with HIV and *M. tuberculosis* occur (WHO, 2005).

The economic impact of this pathogenic synergy is particularly great because HIV disproportionately affects persons during the most productive years of their lives. Around 80% of new cases of TB occur in 22 high burden countries. The incidence of tuberculosis excluding TB/HIV co-infection, increased by 0.6% in 2002-2003. The increase in incidence was 1% when TB/HIV co-infection was included. There has been an increase in incidence, prevalence and death from TB cases in eastern and western parts of Africa. However a decrease in prevalence and death from TB was reported in other regions (WHO, 2005).

Human immunodeficiency virus (HIV) infection accounts for much of the recent increase in the global tuberculosis burden. Worldwide, an estimated 11% of new TB cases in 2000 were infected with HIV, with wide variations among regions: 38% in sub-Saharan Africa, 14% in more developed countries, and 1% in the Western Pacific Regions. The increase in tuberculosis incidence in Africa is strongly associated with the prevalence of HIV infection (Corbett, 2002).

Ethiopia ranks eighth among the world's 22 countries with a high tuberculosis burden. According to the WHO Global TB Report 2006, the country had more than 267,000 new TB cases in 2006. The country has successfully expanded DOTS (Directly Observed Treatment Short course) with health service coverage of 75%. Nevertheless the incidence of all cases of TB is estimated to be 353/100,000 and smear positive TB to be 155/100,000. Mortality of TB patients in the country is estimated to be 79/100,000 and TB is the first cause of hospital deaths comprising 27% of all total hospital deaths. TB is also the third cause of hospital admissions (accounting for 9.4% of all admissions) and the fourth cause of outpatient department (OPD) morbidity accounting for 3.7% of all OPD visits. TB cases are likely to increase as Ethiopia's HIV/AIDS epidemic expands and as nearly one-quarter of adult TB cases are HIV-positive. TB/HIV co-infection rate between the ages of 15-49 years is estimated to be 21% and multi-drug resistance (MDR-TB) (resistance at least to Rifampicin and Isoniazid) is 1.4%. The success rate of treatment in the country was reported to be 76% in 2002 (TLCT, 2002; WHO, 2005).

1.3. Global Epidemiology of HIV/ AIDS

It has been estimated that at least 10.7 million persons were co-infected with HIV and *M. tuberculosis* in 1997, and those HIV-1-infected patients represent 8% of the worldwide total of TB cases (Dye *et al*., 1999). More than 30% of TB cases in Africa are also co-infected by HIV (Dye *et al*., 1999). During HIV infection, an increased risk of developing TB has been found in males, and in those living in areas such as sub- Saharan countries, where malnutrition and social deprivation are factors (Selwyn *et al*., 1992).

The number of people living with HIV/AIDS has risen from around 8 million in 1990 to nearly 40 million in 2006, and is still growing. Around 63% of people living with HIV are in sub-Saharan Africa. During 2006, around four million adults and children became infected with HIV, the virus that causes AIDS (WHO, 2006). The year also saw around three million deaths from AIDS, despite recent improvements in access to antiretroviral treatment (WHO, 2006).

Most TB cases are found in southern Asia (Dye *et al*., 1999), the incidence of TB does not vary according to the route of HIV transmission, but the risk of developing TB after exposure to an infectious contact has been estimated to be 5–15% per year in HIV- infected patients as compared to 5–10% during the lifetime of HIV-negative patients (Raviglione *et al*., 1997). The TB mortality rate also increased at the beginning of the HIV pandemic in areas hyper-endemic for both HIV and TB, particularly in Africa and Asia, where TB can develop early after exposure to an infectious contact even in non-severely immunocompromised patients.

According to the fifth Report of AIDS in Ethiopia, HIV/AIDS is claiming an estimated toll of as many as 115,000 lives a year and it is believed that this annual death toll could reach 200,000 unless effective prevention and treatment strategies are made available. Thirty percent of adult (15-49 years of age) deaths have been attributed to HIV/ AIDS, while the epidemic has resulted in an estimated 539,000 orphans (MoH, 2006).

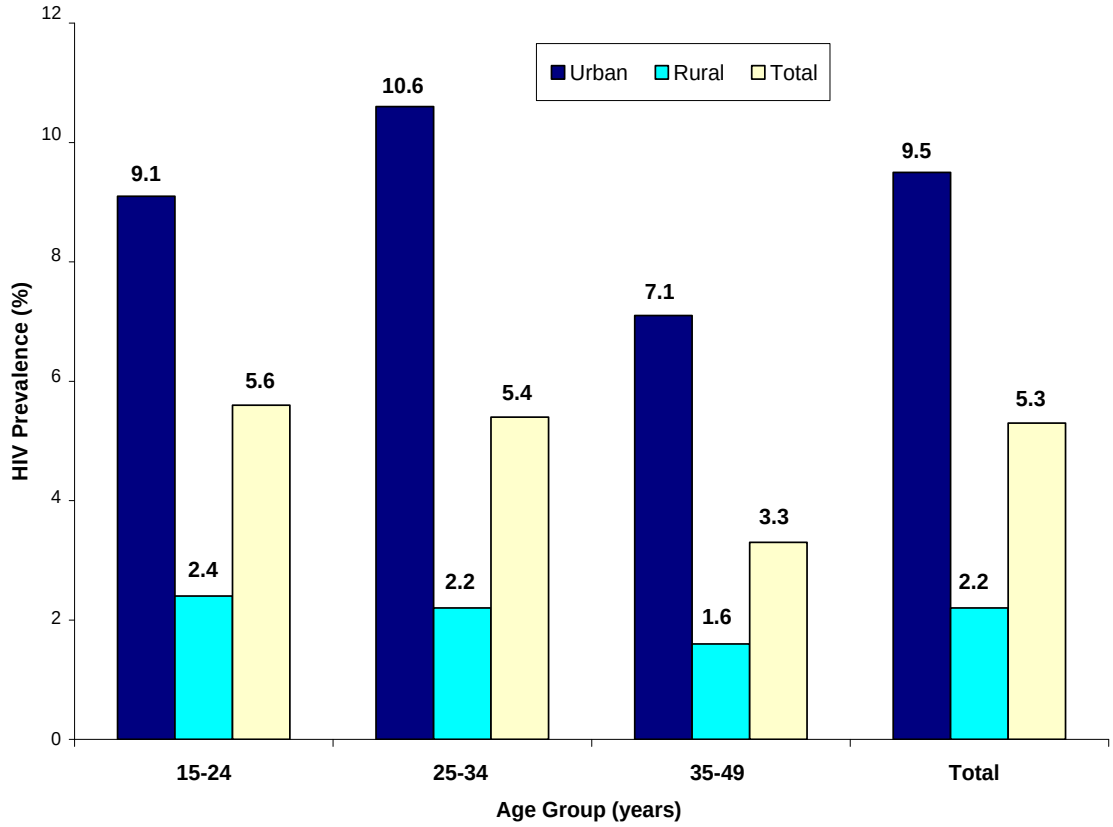


Figure 1.1. *HIV Prevalence by Age Group and Site Setting (AIDS in Ethiopia Sixth Report, MoH, 2006).*

Trends in the epidemic situation show a marked difference between urban and rural areas. The HIV epidemic appears to reach its peak and stabilizes at a high prevalence level in urban areas, while it is increasing gradually although at a much lower level and reduced rate in the rural Ethiopia (MoH, 2006).

1.4. TB Control Program in Ethiopia

Ethiopia is a federal state with an estimated population of 78 million and is divided into 9 regions and has two chartered cities. One of these regions is the Oromiya region. The region is divided into different zones and West Arsi zone is the biggest zone and an estimated population size of 3.5 million. The zone has its own Health Bureau and there is a Zonal Health Department, 1 hospital, 10 health centers and 70 health posts under it.

The 10 health centers are diagnostic and treatment units while the 70 health posts are used as treatment units only. All regional and central hospitals serve as diagnostic and referral centers. In all treatment and diagnostic units, one physician trained on TB is available and a nurse is in charge of the overall activities of the TB clinic. The physician in charge makes the decision on treatment initiation for smear negative and extra-pulmonary TB cases.

Based on the available data from the zonal Communicable Disease Control Team (CDCT) of the Regional Health Bureau, the number of cases of tuberculosis in the West Arsi zone has been on the rise. Case notification of all forms of TB was around 7,689 in 2002/2003. Smear positive pulmonary TB, smear negative pulmonary TB and extra-pulmonary TB cases comprise nearly 1/3 each. There were 2,688 patients on DOTS treatment with a treatment success rate of 78.1%. The above figures might not reflect the actual picture of the prevalence of TB. These figures are estimates based on the number of patients who visit health facilities seeking medical attention. It is estimated that about 50% of tuberculosis cases do not get detected at the National level (WHO, 2005).

1.5. FIRST LINE ANTI-TB DRUGS

There are five first line anti-TB drugs used for tuberculosis treatment. These include Isoniazid (INH), Rifampicin (RMP), Pyrazinamide (PZA), Streptomycin (STM) and Ethambutol (EMB). INH is a pro-drug that is activated to different oxygen free radicals and organic radicals which then attack different targets. The primary target of inhibition is the cell wall mycolic acid synthesis pathway, where enoyl ACP reductase (*Inh*A) was identified as the target of INH inhibition (Zhang *et al.*, 1992; Heiym *et al.*, 1993). This is an important enzyme for the synthesis of the cell wall. The active species for *Inh*A inhibition has been found to be isonicotinic acyl radical, which reacts with NAD to form INH-NAD adduct and then inhibits the *Inh*A enzyme (Rozwarski *et al.*, 1998). The reactive species produced during INH activation could also cause damage to DNA, carbohydrates, and lipids (Zhang, 2005) and inhibit NAD metabolism (Winder and Collins, 1968).

Isoniazid and Rifampicin have high early bactericidal activity on rapidly proliferating bacilli (Mitchison, 2000). These drugs account for killing of approximately 95% of bacilli in the first two days of treatment. These drugs, therefore, effectively prevent transmission of tuberculosis during early weeks of treatment. However, each drugs relatively less efficacious on semi-dormant bacilli.

Rifampicin is an efficacious drug that binds to the β-subunit of RNA polymerase and inhibits the process of transcription (Zhang, 2005).

Pyrazinamide on the other hand, is a pro-drug that is activated to pyrazinoic acid by Pyrazinamidase (Konoko *et al.*, 1967). The mechanism of action for PZA is not fully understood but one possible mechanism is believed to be inhibition of fatty acid synthase I (FASI), an enzyme involved in fatty acid biosynthesis (Zimhony *et al.*, 2000). Another mechanism suggested is cytoplasmic acidification and has effect on membrane energy metabolism (Zhang, 2005). Rifampicin and Pyrazinamide are drugs that are particularly effective in eliminating special subpopulations of bacilli such as those in semi-dormant states and in acidic environment respectively. These drugs have the highest sterilizing effect and

often kill the last few bacilli during the course of treatment. Therefore, they effectively shorten duration of treatment (Mitchison, 2000).

Streptomycin, an aminoglycoside antibiotic, primarily interferes with protein synthesis by inhibiting initiation of mRNA translation, facilitating misreading of the genetic code and damaging the cell membrane (Zhang, 2005). The site of action is in the small 30S subunit of the ribosome, specifically at ribosomal protein S12 (*rps*L) and 16S rRNA (*rrs*) in protein synthesis (Garvin *et al.*, 1974).

Ethambutol is one of the first-line anti-TB drug which inhibits cell wall synthesis. EMB inhibits arabinosyl transferase, an enzyme important for cell wall synthesis (Telenti *et al.*, 1997). It acts on rapidly proliferating organisms and less effective drug compared to other drugs. All first-line anti-TB drugs are said to be bacteriocidal except Ethambutol which is bacteriostatic (Zhang, 2005).

The treatment regimen that is given for smear positive category I patients is two months of treatment with RMP, INH, PZA with STM or EMB, followed by six months of EMB and INH. In the continuation phase the semi-dormant bacilli are more common than in the rapidly proliferating bacilli (Mitchison, 2000). There are also recommendations to replace EMB with RMP in the continuation phase for enhanced efficacy. This will also shorten the duration of treatment by two months (Mitchison, 2000).

1.6. ANTI-TB DRUG RESISTANCE

Drug resistance is defined as a decrease in susceptibility of an isolate to a sufficient degree to be reasonably certain that the strain concerned is different from a wild strain that has never come in contact with the drugs (WHO, 1997). Generally, when one percent or more of an isolates are resistant to an anti-TB drug, therapeutic success is less likely to occur. The isolate is then considered resistant to the drug (Canetti *et al.*, 1963).

1.6.1. Mechanism of Anti-TB Drug Resistance

Resistance of *M. tuberculosis* to anti-TB drugs is a man-made amplification of a natural phenomenon. Wild strains of *M. tuberculosis* that have never been exposed to anti-TB drugs are almost never resistant, though natural resistance to specific drugs has been documented for *M. bovis* and other atypical mycobacteria (WHO/IUATLD, 2000). During bacterial multiplication, resistant bacilli evolve through spontaneous mutation and with defined frequency. Mutation that results in INH resistance to *M. tuberculosis,* for example, occurs at a rate of 10^{-7} to 10^{-9} per cell division and leads to an estimated resistance of 1 in 10^{6} bacilli in drug-free environment (Lambregts-van Weezenbeek *et al.*, 1998). Thus, resistant organisms (mutants) evolve in the absence of antimicrobial exposure. However, these mutants are diluted within the majority of drug-susceptible mycobacteria since bacillary populations greater than 10^{7} are common in the lung of TB patients (Canetti, 1965). The presence of a single antimicrobial drug, to which mutation has been developed, provides the selective pressure that helps resistant organisms to predominate. This process could especially be common in situations where there is a large load of bacilli such as in cavities (Howard *et al.*, 1949; Howlett *et al.*, 1949).

Exposure to a single anti-TB drug due to irregular intake, poor drug quality, inappropriate prescription and/or poor adherence to treatment could result in functional monotherapy.
This will suppress the growth of bacilli susceptible to that drug but permits the multiplication of drug resistant organisms (Crofton and Mitchison, 1948; Mitchison, 1950). This phenomenon is called acquired drug resistance (secondary drug resistance). Subsequent transmission of such bacilli to other persons may lead to disease which is drug-resistant from the outset, a phenomenon known as initial resistance (Figure 1.2). When this process is repeated through the same process, multiple drug or multidrug-resistance will be developed. Every drug effective against TB is bound to select for resistant bacilli (Canetti, 1965).

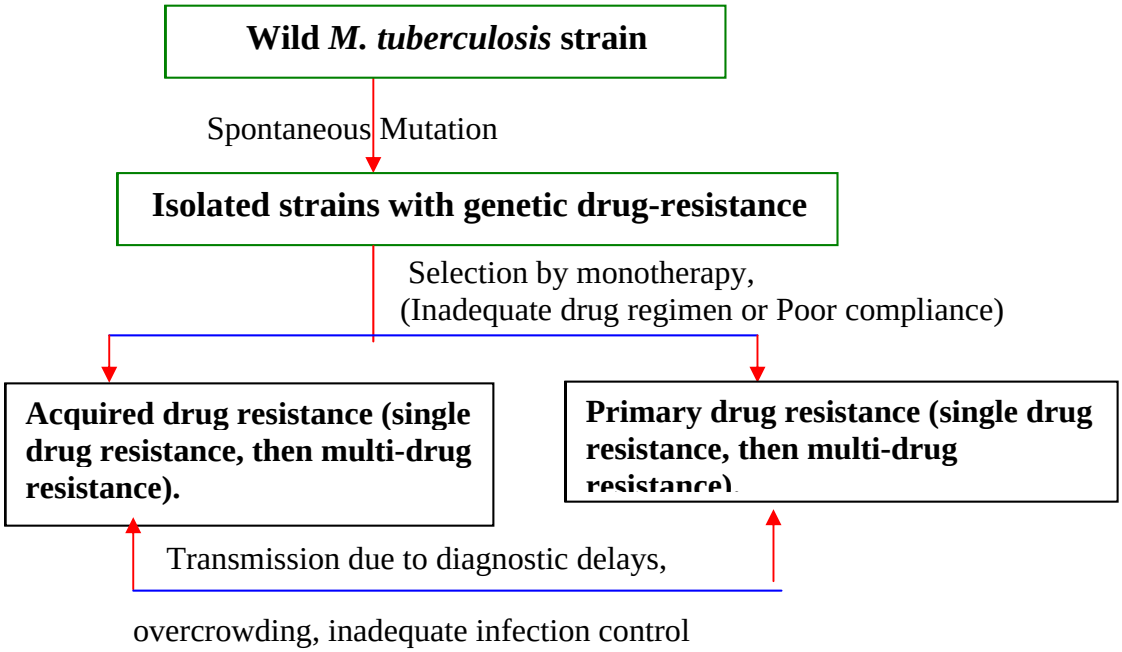


Figure 1.2. Development and spread of drug and multi-drug resistant tuberculosis (Global project on anti-TB drug resistance surveillance, 2000)

1.6.2. Molecular Genetic Basis of Anti-TB Drug Resistance

The genetic basis of resistance development to most anti-TB drugs are established. Resistance to Rifampicin results from missense mutations in the *rpo*B gene, which encodes the β–subunit of RNA polymerase (Miller *et al.*, 1994). RNA polymerase is an essential enzyme with five subunits that catalyzes the process of transcription. Mutation in *rpo*B gene results in resistance by decreasing Rifampicin-binding affinity (Table 1.2.). These mutations are centered on a region between codons 507 and 533 of the *rpo*B gene. More than 35 resistance alleles have been identified in this region (Musser, 1995; Gingeras *et al.*, 1998). Mutation leading to Rifampicin resistance is rare and occurs at a rate of 10^{-10} per cell division. This results in resistance of 1 in 10^{8} bacilli in a drug-free environment (David, 1970).

Isoniazid is a drug that inhibits the synthesis of mycolic acids in *M. tuberculosis*. It is a pro-drug that is activated by the enzyme catalase peroxidase. *Kat*G, which encodes catalase peroxidase, *inh*A, *ahp*C and *kas*A are genes involved in resistance to Isoniazid (Table 1.2.).

Among these, *kat*G is the most frequent gene responsible for Isoniazid resistance. Approximately 90% of resistant isolates contain mutations in *kat*G with the majority localized to codon 315 (Zhang *et al.*, 1992). Catalase peroxidase, expressed by a mutant *kat*G, either has low affinity to INH or is produced in lower quantity, or both (Heym *et al.*, 1997). Such mutations were found in INH resistant strains, and one particular substitution in the codon 315, AGC→ACC (Ser →Thr), was reported as the most frequent, apparently providing the optimal balance between the decreased catalase and sufficiently high peroxidase activities of *Kat*G (Ramaswami and Musser, 1998). This mutation was reported to be associated with intermediate or high levels of resistance to INH (1 to 10 µg/ml) (Marttila *et al.*, 1996; Van Soolingen *et al.*, 2000), though it has recently been suggested that the *kat*G315 alteration alone may not always be clinically significant (Abate *et al.*, 2001).

A missense mutation of the *inh*A gene which encodes Enoyl-ACP reductase, an enzyme involved in the mycolic acid biosynthetic pathway, also causes INH resistance. About 20-34% resistant isolates have mutations in the promoter region of *inh*A, either alone or in combination with *kat*G. Isoniazid resistance following isolated *inh*A mutation is rare (Hongthiamthong *et al.*, 1994). Mutations have also been detected in the *ahp*C promoter region of Isoniazid resistant isolates. However these mutations are always observed to occur together with mutations in *kat*G (Riska *et al.*, 2000). There are also studies suggesting that mutations in *ahp*C are rather compensatory and enable INH resistant strains survive the oxidative stress in macrophages.

Pyrazinamide is activated to an active form, pyrazinoic acid, by pyrazinamidase, an enzyme encoded by *pnc*A. Mutation in *pnc*A is the major cause of Pyrazinamide resistance. Between 72% and 98% of Pyrazinamide resistance in clinical isolates is correlated with mutations scattered through out *pnc*A coding region and 11 promoter regions. The rate of mutation for Pyrazinamide is 10^{-3} with a probability of getting 1 resistant organism out of 10^{6} bacilli (Scorpio and Zhang, 1996).

Streptomycin is an antibiotic that interferes with prokaryotic protein synthesis through binding to ribosomal protein. Resistance to it is mainly due to mutations in the *rps*L locus encoding the S12 ribosomal protein. Approximately 60% of Streptomycin resistant clinical isolates show

*rps*L mutation. Resistance to streptomycin is mainly due to mutation in the *rps*L locus encoding the S12 ribosomal protein. The drug binds to the 16S rRNA, interferes with translation proofreading, and thereby inhibits protein synthesis (Bottger, 1999). Streptomycin resistance in *M. tuberculosis* is associated with substitution of amino acid 43 or 88 of ribosomal protein S12. Amino acid 43 and 88 replacements were identified in strains from widespread geographic localities (Bottger, 1999). About 10% of resistant strains have mutations in 16S ribosomal RNA which is encoded by *rrs* gene (Bottger, 1999). The mutation rate for Streptomycin is 10^{-8} resulting in 1 STM resistant bacillus out of 10^{7} bacilli.

Ethambutol is a drug which targets cell wall synthesis. The major mechanism of acquisition of resistance to the drug is associated with point mutations in the *emb*CAB operon. This operon is composed of three organized genes encoding different arabinosyl transferases that are involved in cell wall synthesis. In particular, amino acid replacement at position 306 of *emb*B has been shown in many studies to be present in Ethambutol-resistant and not in susceptible organisms. The mutation rate for Ethambutol is 10^{-7} with a resistance of 1 out of 10^{5} bacilli (Ramaswamy and Musser, 1998). Different target anti-TB drugs show different mutation rate thus different drug

1.6.3. Multi-Drug regimen and drug resistance

Almost all TB patients have a bacillary load that could result in the selection of drug resistant bacilli if exposed to monotherapy (Canetti, 1965). Administration of INH alone, for example, to TB patients could result in selection of an INH resistant strain. On the other hand, if INH and RMP are administered together, bacilli that would be resistant to INH will be killed by RMP. The same would be true to the other way also. Therefore, development of resistance to anti-TB drugs could be prevented (Canetti, 1965).

Presence of different semi-dormant bacilli and variation in pH at the infection site imply the need for drugs with different roles that could cover all subpopulations of bacilli (Mitchison, 1998). Therefore, no single drug is efficient for very long in treating TB, emphasizing a need to combine anti-TB drugs.

Table 1.1. Molecular genetic target of Anti-TB drug resistance and their rate of mutation

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

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

Multiple drug resistance due to spontaneous mutation, occurring at the same time, is practically impossible unless mutations are acquired sequentially. This is because of absence of a single gene mutation involved in acquisition of multiple drug resistance (WHO, 1997). The probability of spontaneous mutation causing multiple resistance is then a product of the probabilities of mutations to individual drugs. For instance, spontaneous mutation resulting in combined resistance to INH and RMP is developed at a rate of 1 in 10^{14} (10^6x 10^8) (Iseman and Madsen, 1989). This implies that a minimum bacterial load of 10^{14} bacilli is required for spontaneous mutation to both INH and RMP to occur at the same time. This type of bacterial load could not be seen even in a cavitary TB (Iseman and Madsen, 1989).

1.6.4. Drug Concentration and Development of Resistance

Research findings indicate that poor quality drugs, especially fixed dose combination (FDC) anti-TB drugs, attain lower drug concentrations (Shishoo *et al.*, 2001). Rifampicin, a drug believed to be the cornerstone of TB treatment, is the one frequently observed to attain lower serum concentration. This is believed to be due to poor water solubility of the drug and its close contact with INH in an acidic environment of the stomach (Shishoo *et al.*, 1999). Rifampicin was shown to change into 3-formyl rifamycin SV, an insoluble compound incapable of absorption, at a lower rate. Presence of INH in acidic environment will substantially increase the rate of conversion of RMP into 3-formyl rifamycin SV.

The concentration of the antibiotic has an important role in the rate of mutation to antibiotic resistance. For instance, the rate of selection of low-level quinolone-resistant mutants in *P. aeruginosa* can range from 1.2×10^{-6} to 4×10^{-10}, depending on concentration of the quinolone used for selection (Kohler *et al.*, 1997). A phenotype of increased antibiotic resistance may arise from mutations in different genes. These mutations, however, may provide quite different levels of antibiotic resistance. In fact, heterogeneous expression of antibiotic resistance (even with changes in the same target gene) is a well-known phenomenon (Figueiredo *et al.*, 1991). At low antibiotic concentrations, mutations in any of those genes can effectively protect the bacteria from the action of the antibiotic and thus be selectable. However, once the antibiotic concentration rises, the number of selectable mutants decreases.

Another important point is that the probability that a specific type of mutant will emerge is expected to peak at antibiotic concentrations close to the minimal inhibitory concentration (MIC) for the organism (Baquero and Negri, 1997). For instance, a specific antibiotic concentration may be sufficient to decrease the growth rate or to suppress the original ancestor population but may not be sufficient to affect the emerging resistant variant population (MIC for susceptible population). Beyond this level, antibiotic concentrations may be able to reduce or suppress in an equivalent way the growth of both susceptible and resistant populations, and therefore, no selection for the variant is expected to occur (Baquero and Negri, 1997).

Almost all anti-TB drugs achieve much higher serum concentrations than the MIC for drug sensitive TB sub-population in usual circumstances (WHO, 1997). As the drug concentration falls significantly towards MIC of the drug susceptible population, the selection of drug resistant sub-population will be maximal. On the other hand, as the selective effect of the drug may depend on the time of exposure, this period of time may be critical to yield one or another mutation rate (Martinez and Baquero, 2000).

Finally, the dynamics of the antibiotic action on the bacterial cell may modify the mutation rate. If the bacterial population is not killed effectively by a given antibiotic concentration, the cells are maintained under stress, which may increase the mutation rate (Shapiro, 1997). On the contrary, some potentially effective resistance mutations may have a phenotypic lag; that is, the resistance phenotype is not immediately evident after mutation but appears some time later. If

at this time the bacterium is rapidly killed by the drug, the resistance phenotype will never arise (Martinez and Baquero, 2000).

1.7. METHODS OF DIAGNOSING DRUG RESISTANT TB

Drug susceptibility testing can be done either directly using an acid-fast positive clinical sample or indirectly using mycobacteria isolated in culture media. The traditional method using agar-base, egg-base or liquid media follows either of the three different varieties of susceptibility testing; the proportion method, the absolute concentration method and the resistance ratio method (Canetti *et al.*, 1969; Campos *et al.*, 2003).

In the proportion method, resistance is defined as more growth in drug-containing media compared to the growth on drug-free control media containing 1 in 10 diluted bacterial suspensions. Therefore, bacterial suspensions and dilutions are prepared and inoculated on drug-free and drug-containing media. When the growth is visible, comparison will then be made between the two media.

In resistance ratio method, a ratio is obtained by dividing the minimum inhibitory concentration (MIC) of isolate with the MIC of a susceptible reference strain. The clinical isolate is defined as resistant if the ratio is 8 or more and susceptible if the ratio is 2 or less. A test will be repeated if an intermediate result (i.e. ratio between 2 and 8) is obtained. An intermediate result on a repeat test can be considered resistant.

In absolute concentration method drug-resistance is defined as the growth of 20 colonies or more on media containing a defined concentration of the test drug.

These indirect tests could take 7 to 12 weeks. However rapid methods like BACTEC, Mycobacteria Growth Indicator Tube (MGIT), ESP Myco, and MB/BactT have already been developed or are under development for commercial use. Additionally, different molecular techniques which rely on the detection of mutation in the regions of gene (s) associated with drug action could also be useful for rapid diagnosis of drug resistance (Heifets and Cangelosi, 1999).

1.8. GLOBAL EPIDEMIOLOGY OF DRUG RESISTANT TB

The global distribution of drug resistant TB was poorly defined until recently. In 1999, the International Union Against Tuberculosis and Lung Disease (IUATLD) and WHO launched the global Project on Anti-tuberculosis Drug Resistance Surveillance. The first report was between 1994 and 1997 based on data collected from 35 countries on 5 continents. Drug resistance was detected in all countries which indicate that it is a global problem. Additionally, 9.9% of TB patients had drug resistant isolates and a median of 1% of them had MDR. But countries such as the former Soviet States, the Dominican Republic and Argentina were of particular concern for high levels of drug resistance (WHO, 1997).

In the second report, 72 countries were included in the survey in 1994 to 2000. The prevalence of resistance to more than one drug ranged from 1.7% in Uruguay to 36.9% in Estonia, with a median prevalence of 10.7%. The overall median prevalence of MDR was again 1%, the level of MDR-TB among new cases in 9 regions was greater than 3%. No cases of MDR-TB were reported in some countries (WHO/IUATLD, 2000).

In the third report of anti-TB drug resistance surveillance, 77 settings were surveyed in 1994 to 2002. It was noticed that the median prevalence of drug resistance was 1.1% (0-14.2%) whereas the median prevalence for any type of resistance was reported to be 10.2% (0%-57.1%). Again most regions had a lower rate of MDR-TB prevalence. On the other hand in 10 regions MDR-TB prevalence was greater than 6.5 % (WHO/IUATLD, 2004).

In Botswana, anti-TB drug resistance has shown a significant upward trend. Resistance against any TB drug was reported as 3.7% in 1995 increased to 6.3% in 1999 (WHO/IUATLD, 2000) and then to 10.4% in 2002 (WHO/IUATLD, 2004). The country is known for its strong DOTS program. Studies in the country have shown no association to exist between drug resistant TB and HIV infection even if the country has a high HIV prevalence (Kenyon, 1999).

1.9. PROBLEMS ASSOCIATED WITH DRUG RESISTANT TB

Due attention should be given to anti-TB drug resistance since the situation causes different problems such as failing on treatment, relapse after cure and increased likelihood of transmission are some of the risks that affect the effectiveness of TB control programs (Noeske and Nguenko, 2002).

1.9.1. Treatment Failure and Death

Patients with drug resistant TB are observed frequently to fail on treatment (Noeske and Nguenko, 2002). Patients with MDR-TB specifically were 15 times more likely to fail on treatment than patients with drug sensitive TB. Death was also significantly higher in patients harbouring MDR-TB. Particularly, life span of HIV patients with drug resistant TB is short (Fischl *et al.*, 1992). Treatment failure was also more common in other types of resistance than MDR. An approximately linear increase in the likelihood of treatment failure was observed as the number of drugs to which the strains were resistant increased (Espinal *et al.*, 2000).

There are also reports indicating that around 90% of re-treatment cases failing on the standard re-treatment regimen have MDR-TB (Espinal *et al.*, 2000). Moreover, around 65% of patients with no initial resistance or other types of resistance than MDR will acquire MDR at failure. In general failure cases on 2SRHZ/6HE had a high prevalence of MDR (80%), half of which was primary drug resistance and the remaining half was acquired (Quy *et al.*, 2003).

1.9.2. Relapse

Relapse after cure was observed more frequently in patients with drug resistant TB compared to patients with drug sensitive TB. An estimated 77% of patients with at least one anti-TB drug resistance will develop relapse (Quy *et al.*, 2003). Among treatment relapse cases, MDR was much less common (8%, all of them acquired) compared to the treatment failures. Combined primary Isoniazid and Streptomycin resistance was a strong risk factor for relapse and for acquired MDR among relapse cases (Quy *et al.*, 2003).

1.9.3. Increased Likelihood of Transmission

Smear positive TB patients are more likely to transmit TB to others than smear negative patients (Grzybowski *et al.*, 1975). Under a successful treatment, patients convert to negative smear and stop transmission within an average of two weeks of treatment initiation (Sacks *et al.*, 2001). Factors such as the presence or absence of cavitary disease and drug resistance dictate the rate of conversion to negative smear. TB patients with drug resistance will be slower to smear conversion since sterilization by first line anti-TB drugs is rendered difficult (Sacks *et al.*, 2001). Therefore, these patients will transmit their drug resistant TB to individuals to their vicinity resulting in higher incidence of TB, and specifically of drug resistant ones.

1.10. FACTORS ASSOCIATED WITH DRUG RESISTANT TB

Even though different factors are suggested to be associated with drug resistant TB, there is no consensus for many of the implicated risk factors. Previous treatment with anti-TB drugs and HIV infection are factors usually considered with drug resistant TB.

1.10.1. Previous Treatment

A TB strain previously exposed to anti-TB drugs is likely to undergo selection and to have a greater proportion of drug resistant bacilli (Castello *et al.*, 1980). Hence previously treated patients are more likely to harbour drug resistant strains. Patients who failed on treatment, defaulters and relapses cases after cure fit this category. Previous treatment is a factor shown to be associated with development of drug resistance more consistently (de Melo *et al.*, 2003). A greater proportion of treatment failures, in particular, have drug resistant TB (Quy *et al.*, 2003).

1.10.2. Co-infection with HIV

It has been estimated that at least 10.7 million persons were co-infected with HIV and *Mycobacterium tuberculosis* in 1997, and those HIV-1-infected patients represent 8% of the worldwide total of TB cases (Dye *et al*., 1999). More than 30% of TB cases in Africa are also co-infected by HIV (Dye *et al*., 1999). During HIV infection, an increased risk of developing TB has been found in males, and in those living in areas such as sub- Saharan countries, where malnutrition and social deprivation are factors (Selwyn *et al*., 1992).

There are different reasons, in principle, why anti-TB drug resistance might be common among HIV- infected patients. First, drug resistant *M. tuberculosis* is believed by some authors, for some of the strains, to be less virulent which could cause disease only in immunosuppressed patients (Ordway *et al.*, 1995). The second possibility is that there may be shared risk factors for infection with HIV and drug resistant strains of *M. tuberculosis*, such as hospitalization. This is likely to be the main reason for numerous reported nosocomial outbreaks of MDR among AIDS patients (Breathnach *et al.*, 1998; Moro *et al.*, 1998). Thirdly, treatment of HIV patients may fail because such patients carry a larger number of bacteria and poor immunity that does not assist the drugs (Dye *et al.*, 2002). Finally HIV patients may be subjected to functional monotherapy (Berning *et al.*, 1992).

HIV has been linked to MDR in small-scale outbreaks, such as those originating in institutions (Ritacco *et al.*, 1997). However, there is no evidence that MDR is associated with HIV in sporadic situations and in cross-country comparisons (Eyob *et al.*, 2004). In particular, MDR appears to be uncommon in sub-Saharan Africa, the epicentre of the AIDS pandemic (WHO/IUATLD, 2000).

We do not know at the moment, but XDR-TB is rare. However, WHO estimates that there were almost half a million cases of MDR-TB worldwide in 2004, and MDR-TB usually has to occur before XDR-TB arises. We also know that findings from the only global study carried out so far showed that in some places perhaps as many as 19% of MDR-TB cases were in fact XDR-TB, but this is likely to be uncommon. Wherever second-line drugs to treat MDR-TB are being misused, the possibility of XDR-TB exists. Research is being carried out urgently to find out more.

1.11. FITNESS OF DRUG RESISTANT TB

There had been controversial information on the relative fitness of drug resistant TB organisms to infect and cause disease. Earlier evidence was based on animal studies where Isoniazid resistant strains were observed to be less fit as compared to the susceptible ones (Ordway *et al.*, 1995). Later on, it was observed that close contact to patients with drug resistant and susceptible strains developed infection and disease at a comparable rate (Snider *et al.*, 1985;

Teixera *et al.*, 2001). Yet, other studies in other areas based on fingerprint analysis, have shown significantly reduced clustering of drug resistant TB strains compared to the drug susceptible ones implying less recent transmission (van Soolingen *et al.*, 1999). This could be taken as evidence that drug resistant TB is not as infectious and/or pathogenic as the drug susceptible ones. There are also findings from fingerprint analyses which suggest that drug resistant TB is as transmissible as the sensitive ones (Vukovic *et al.*, 2003).

There are at least two explanations that could reconcile this difference of observations. First, different types of mutations in a single gene could result in a range of fitness (Hailemariam *et al.*, 2004). For example, in an *in-vitro* study, it was demonstrated that a number of *rpo*B mutation types in bacilli showed a range of fitness. Probably one type of mutation may be more prevalent in one locality and other types in other areas. Second, a single strain of drug resistant TB could show a heterogeneous degree of fitness during its course (Sherman *et al.*, 1996). Hence a resistant strain less fit at one point in time may evolve into a competent strain after accumulating compensatory mutations later.

Comparison of the relative fitness of drug resistant and sensitive organisms revealed that some mutations compromise growth and the ability to survive oxidative stress. However, other mutations have minimal *in-vitro* effects (Ordway *et al.*, 1995; Hailemariam *et al.*, 2004). Furthermore, when fitness costs were detected, they were short-lived (Sherman *et al.*, 1996).

Different mathematical models have tried to show the impact of drug resistance on TB control programs (Dye and Espinal, 2000; Dye and Williams, 2000; Dye *et al.*, 2002). One such model assumes a heterogeneous fitness (Cohen and Murray, 2004). In this model it was shown that even when the fitness of drug resistant TB is low and a strong control program is in place, a small subpopulation of a fit drug resistant strain may out compete both drug sensitive and the less fit drug resistant strains. Thus, current epidemiological studies and short-term trends in the burden of drug resistant TB do not provide evidence that drug resistant TB (especially MDR-TB) strains can be contained in the absence of specific efforts (such as DOTS-plus).

1.12. ANTI-TB DRUG RESISTANCE AND TB CONTROL PROGRAMS

Inappropriate treatment of TB in general and functional monotherapy in particular, results in drug resistance. TB control programs should ensure the regular supply of good quality anti-TB drugs, appropriate prescription and adherence to treatment. If a control program can put these conditions in place, TB patients would be treated appropriately. Together with prompt diagnosis, appropriate treatment will in turn guarantee a good TB control program. Therefore, the level of anti-TB drug resistance in a population may be a proxy indicator of the effectiveness of a control program in the area (WHO, 1997; WHO/IUATLD, 2000; WHO/IUATLD, 2004). DOTS is currently well accepted as a TB control strategy. This strategy, according to some TB experts, has a potential to reduce the level of anti-TB drug resistance in areas where it is practiced appropriately (Dye and Espinal, 2000; Dye *et al.*, 2002).

Prevalence of drug resistance and effectiveness of a regional or national program were also compared in the three global surveillance programs of anti-TB drug resistance surveillances. All of the three reports showed countries with well-functioning TB control programs to be associated with lower prevalence of drug resistance (WHO, 1997; WHO/IUATLD, 2000; WHO/ IUATLD, 2004). Countries or regions with poor TB control programs were found to have a prevalence of MDR-TB that was 2.5 fold greater than areas with good TB-control programs.

In general, a high level of primary anti-TB drug resistance is an indication of earlier problems in the control program with transmission of resistant strains. Acquired resistance to anti-TB drugs, on the other hand, is more likely to reflect problems in the ongoing program (Petrini and Sven, 1999). In areas with low prevalence of drug resistance among new cases, DOTS may prevent further acquisition of resistance. However, in patients with drug resistant TB, there could be an "amplifier effect". For example a single drug resistance, if not given particular attention, will lead to double resistance and then to triple resistance and so on (WHO/IUATLD, 2004). Therefore, in areas with already high prevalence of drug resistance, DOTS could not be as effective as in those areas with low prevalence of drug resistance.

1.13. DRUG RESISTANT TB IN ETHIOPIA

Recently, the prevalence of MDR-TB in Ethiopia was shown to be 1.6% (Dr. Eshetu Lemma, unpublished data). Nevertheless, there have been a number of reports patchily done over the years on drug resistance of TB strains isolated from various regions of the country. In 1984, a report on anti-TB drug resistance, probably the first in the country among new TB patients of Addis Ababa, documented a drug resistance rate of 15% for Isoniazid, 5% for Streptomycin and 5% for both Isoniazid and Streptomycin. During this period, there was no resistance reported for Rifampicin and hence no MDR–TB (Lemma *et al.*, 1984).

However, in 1986, the first MDR–TB prevalence among new cases was reported to be 1% (Wolde *et al.*, 1986). Later, a study which included all Health Centres and Hospitals of Addis Ababa in 1998 reported prevalence of MDR-TB of 0.6% (Demissie *et al.*, 2001) (Table 1.3.). In 2001, a study which included one Health Centre and one Hospital in Addis Ababa reported a MDR-TB rate of nearly 2.7% among new cases and 5.3% for new and re-treatment cases combined (Eyob *et al.*, 2004). According to Dawit Asmamaw (MSc Thesis, AAU/AHRI, 2005), the prevalence of MDR-TB among new cases was 0.6% and resistance to RMP, INH, STM and EMB was 1.2%, 13.3%, 16.8 and 3.5% respectively.

In the re-treatment groups of St Peter's Hospital, Addis Ababa, MDR–TB was around 12% according to the study done in 1997 (Abate *et al.*,1998). Later on, in 2003 a study performed again on re-treatment patients of St Peter's Hospital in Addis Ababa revealed the MDR rate to be 26% (WoldeMeskel *et al*., 2005)

***Table 1.2**. Drug resistance among new cases of TB in studies previously conducted in Ethiopia between 1984 to 2001.*

Year *Sampling centre* **Sample Size**	**1981**[a] *TB centre* **182**	**1986**[a] *TB centre* **167**	**1996**[b] *Black lion hospital* **101**	**1998** [a] *health centres & hospitals* **179**	**2001** [a] *a health centre & a hospital* **73**	**2005**[b] health centers & hospitals **173**
Any resist (%)	**14.8**	**15.2**	**14.6**	**12.9**	**17.8**	**21.4**
INH (%)	**14.8**	**12.0**	**8.3**	**8.4**	**9.6**	**13.3**
RMP (%)	**0.0**	**1.0**	**1.9**	**0.6**	**2.7**	**1.2**
STM (%)	**4.9**	**9.4**	**7.4**	**7.3**	**13.7**	**16.2**
EMB (%)	**0.0**	**0.0**	**0.0**	**0.0**	**2.7**	**3.5**
Res. ≥2 drugs						
H+R+…. (%)	**0.0**	**1.0**	**0.9**	**0.6**	**2.7**	**0.6**
H+S+…. (%)	**2.2**	**2.5**	**2.8**	**3.3**	**NA**	**9.2**

[a] *Proportion method using Lowenstein Jensen medium* [b]*Proportion method using BACTEC, NA=not available; 1981=Lemma et al., 1984; 1986=Wolde et al.,1986; 1996=Bruchfeld et al,2002; 1998= Demissie et al.2001; 2004=Eyob et al., 2004 and Dawit Asmamaw, MSc thesis AAU/AHRI, 2005*

1.14. Molecular Epidemiology of TB

The resurgence of tuberculosis around the world has renewed interest in understanding the epidemiology and pathogenesis of this disease. One important advance in the field of tuberculosis research has been the development of molecular techniques that allow the identification and tracking of individual strains of *M. tuberculosis*. This new discipline, the molecular epidemiology methods revolutionized the fields of research, prevention, and control of tuberculosis, allowing the differentiation between strains, assessment of the overall diversity of *M. tuberculosis* complex strains including difference by region and population, and measurement of the prevalence of endemic strains (Van Soolingen, 2001). However, few molecular studies have been conducted in countries with a high incidence of tuberculosis.

1.14.1. SPOLIGOTYPING

Spoligotyping is a newer technique based on the detection of the spacer on DNA sequences in between the direct repeats in the direct repeat locus of the chromosome of *M. tuberculosis* (Kamerbeek, 1997) and spoligotyping is simple and highly reproducible and has been used to distinguish drug resistance strains and *M. tuberculosis* complex.

Spoligotyping is a type of molecular genotyping technique used in surveillance studies to track the transmission of infectious diseases caused by many strains, especially Beijing family genotype strains (Borgdorff *et al.*, 2003). Molecular genotyping methods are being used more and more to track the transmission of infectious diseases. The discovery of genetic markers for *M. tuberculosis* has facilitated the development of large-scale and reproducible fingerprinting methods (van Soolingen**,** 2001), particularly spoligotyping, which has made feasible the simultaneous detection and differentiation of *M. tuberculosis* strains. Spoligotyping is a PCR-based method capable of analyzing the strain-dependent polymorphisms in the *M. tuberculosis* short direct repeat (DR) chromosomal region, which consists of identical 36-bp DRs interspersed with 35- to 41-bp non-repetitive spacer sequences.

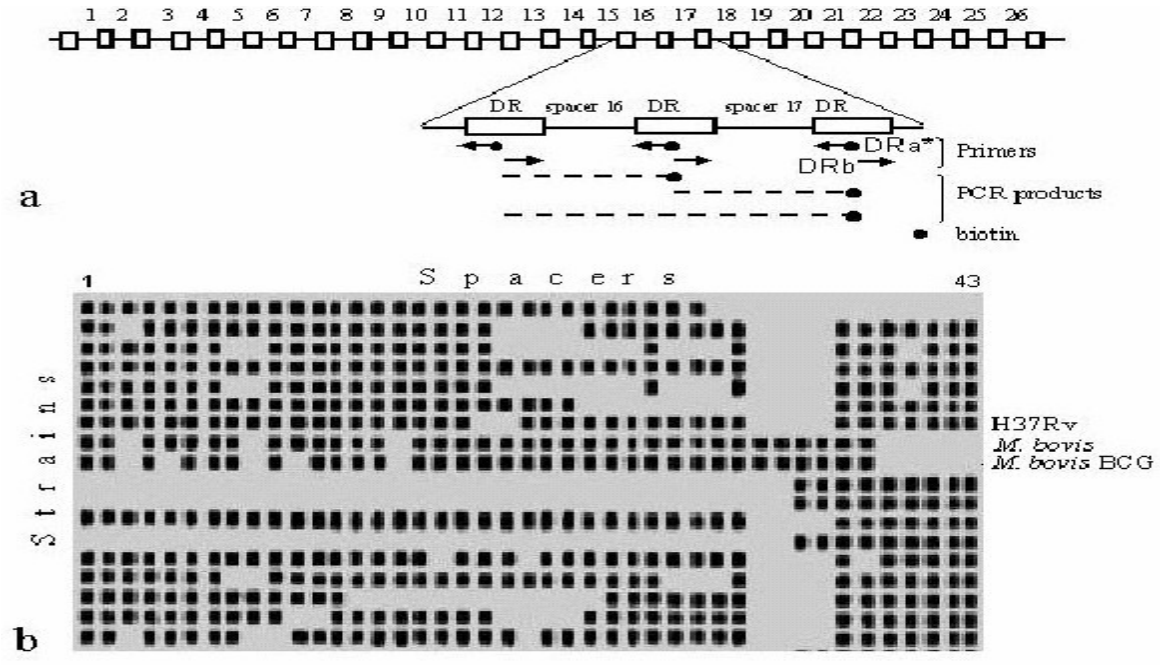


*Figure 1.3. Spoligotyping of M. tuberculosis. a. DR locus structure. b. spoligo profiles of different strains (*Sampson *et al., 2001).*

The direct variable repeat (DVR) spacer oligonucleotide typing technique (Kamerbeek, 1997) detects DNA polymorphism within the direct repeat (DR) locus of *M. tuberculosis* complex organisms. The DR locus contains multiple DVRs that consist of well-conserved 36-bp DRs interspersed with spacer sequences (spacers) 34 to 41 bp long. The order of the DVRs is strongly conserved in the various isolates (van Embden *et al*., 2000). Polymorphism in this region appears to comprise mainly the presence or absence of single, discrete DVRs or stretches of contiguous DVRs (van Embden *et al*., 2000). Differentiation of strains is based on the presence or absence of the spacers in a hybridization pattern (spoligotype), and this has been exploited for the study of the epidemiology of tuberculosis.

Furthermore, the polymorphism obtained by spoligotyping of the IS*6110* low-copy-number *M. tuberculosis* complex isolates proved to be superior to the polymorphism obtained by IS*6110*-associated RFLP (Wilson *et al.*, 1998).This technique has gained widespread acceptance because it is a simple, rapid, and robust method. Results can be expressed in a simple digital format and are easier to compare and store in comparison with those of other available techniques. Thus, it has been extensively applied, alone or in conjunction with other techniques, for tracking epidemics (Wilson *et al.*, 1998); for the description of highly prevalent families such as the Beijing family (van Soolingen *et al*., 1995), multidrug-resistant strain W-Beijing (Bifani *et al*., 1999), to study global epidemiology.

2. Research Gap

Drug resistant TB, particularly MDR-TB is difficult to treat and is associated with high mortality (Espinal *et al.*, 2000). Tuberculosis control programs could be rendered ineffective as a result of development of a high prevalence of drug resistance. On the other hand, drug resistance is the result of a poor TB control program. Therefore, drug resistant TB could be either a cause or an outcome of a poor TB control program and its prevalence could indicate degree of effectiveness of the program.

Drug resistance surveillance activities have not been carried out in the Shashemene area, Southeast Ethiopia. Most of the studies carried out in the central part of the country lack reasonable National representativeness and their primary objective was not determination of drug resistance prevalence. Moreover, it is difficult to compare the different studies and identify a trend since the studies used different methodologies, patient populations, geographical areas (towns) and health facilities. Despite these limitations, the findings were suggestive of an increasing trend of anti-TB drug resistance in the study area. Recently, the prevalence of MDR-TB in Ethiopia was shown to be 1.6% (Dr. Eshetu Lemma, unpublished data).

Shahshemene is one of the known locations with high prevalence of TB and HIV in Ethiopia. The TB-HIV co-infection rate in the age group of 15-49 years was estimated to be 47% in the area (MoH, 2004). Tuberculosis is the leading cause of death in hospitalized patients. However, it has not been investigated whether MDR-TB contributes to the reported mortality.

Therefore, to evaluate the current prevalence of anti-TB drug resistance and its relation with HIV infection by considering appropriate representation of TB patient population in the study area. We performed a health facility based survey of drug resistant and molecular epidemiology of *Mycobacterial* isolates from newly diagnosed TB patients in the Southeast Ethiopia and the strains of *M. tuberculosis* variation among HIV positive and HIV negative TB patients in the study area.

3. Hypothesis:

The prevalence of multi-drug resistance in newly diagnosed TB cases among HIV positive and HIV negative TB patients in the Shashemene area, Southeast Ethiopia is greater than 1.6%.

4. Objectives

General objective:

To determine the anti-TB drug susceptibility pattern of *M. tuberculosis* strains among smear positive pulmonary TB patients in the Shashemene area, Southeast Ethiopia.

4.2. Specific Objectives:

1. To determine the prevalence of any type of anti-TB drug resistance among smear positive pulmonary TB patients with and without HIV co-infection.

2. To determine the prevalence of single, multi-drug and multiple anti-TB drug resistance among smear positive pulmonary TB patients with and without HIV co-infection.

3. To determine if there is an association between drug resistant TB and HIV infection.

4. To further characterize INH and STM drug resistant *Mycobacterial* isolates from pulmonary TB patients with molecular techniques using mutation analysis.

5. METHODOLOGY

5.1. Study Design

This is an institution based cross-sectional survey. The characteristic to be surveyed is proportion of drug resistance among newly treated smear-positive pulmonary TB patients with and without HIV infection. PCR-based mutation analyses were done for INH and STM drug resistance strains by considering *Kat*G315 and *rps*L43 genes respectively. Drug resistance pattern compared among HIV positive and HIV negative TB patients in this study.

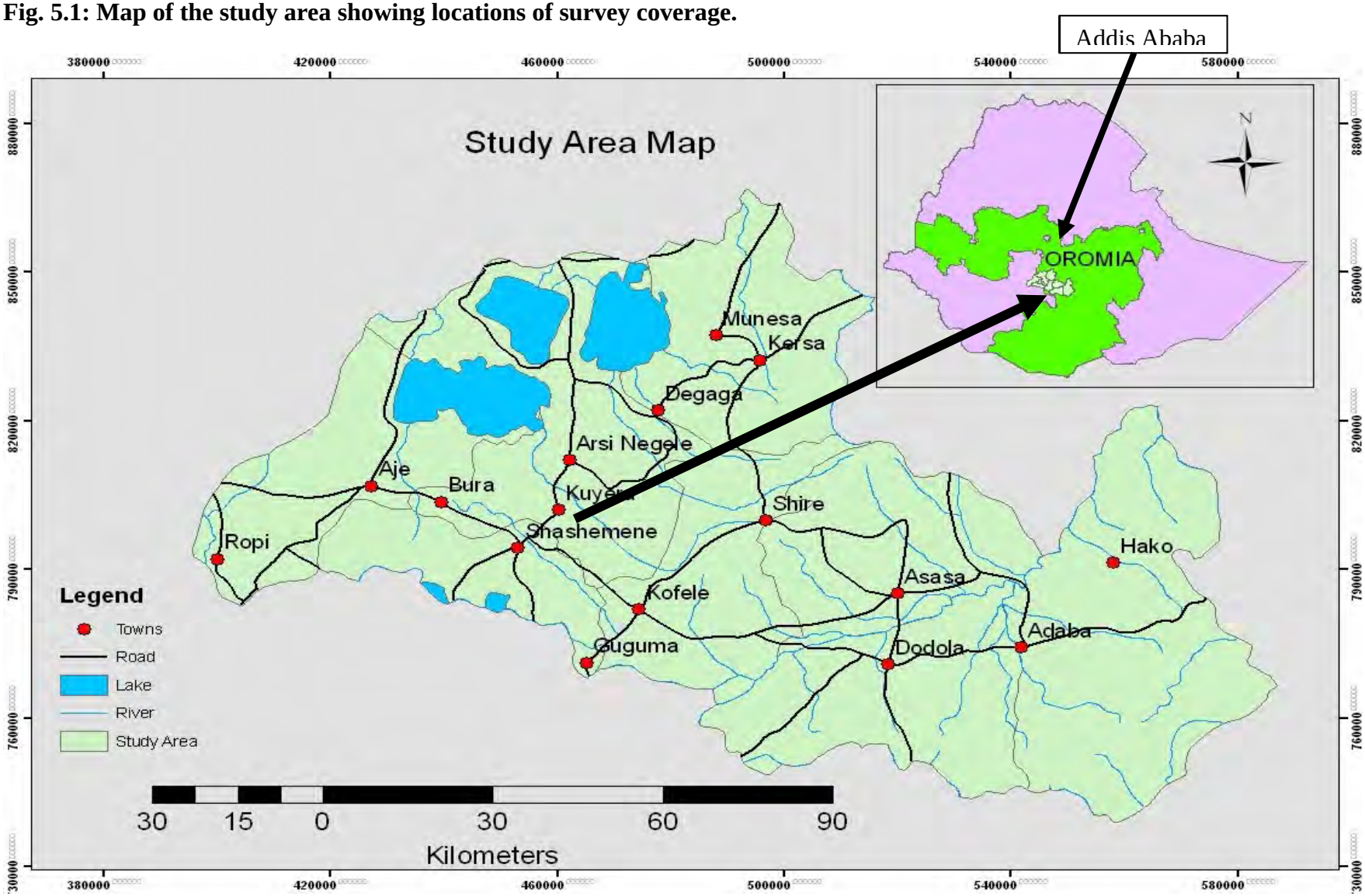
5.2. Study area

This study was performed in Shashemene area, Southeast Ethiopia, at the Health Centers of Arsi-Negelle, Wondo Genet, Aje, and Shashemene and Shashemene General Hospital. The study area includes both Oromia region and Southern Nations Nationalities and Peoples (SNNP) Regional States (Figure 5.1). The estimated population of the study area was about 3.5 million and it included the population in and around the major roadside urban center of Shashemene town and the adjacent rural towns of Wondo Genet, Aje, and Arsi- Negelle. Most of the diagnosis and almost all TB treatment in the area is provided through the DOTS TB clinics in the four Health Centers and the single Hospital in these towns.

5.3. Study population

All newly diagnosed consenting, adult ($\geq$ 18 years), smear-positive pulmonary TB patients with and without HIV co-infected with TB residing in the Shahemene area (Southeast Ethiopia) and visiting Shashemene General Hospital, Shashemene, Arsi- Negelle, Aje and Wondo Genet Health Centers between May 2006 to December 2006, were asked to participate in the study. Those who gave written Informed Consent and fulfilled the inclusion and exclusion criteria were enrolled into the study.

Fig. 5.1: Map of the study area showing locations of survey coverage.



5.4. Sample size and Sampling strategies

Assuming a drug resistance prevalence of 7% in HIV negative TB patients, 20.3% in HIV positive TB patients and HIV/TB co-infection rate of 45% based on Demissie in 2001, the sample size was calculated using the formula [$N= P_1 (1- P_1) + P_2 (1- P_2) / (P_1- P_2)^2 (Z_\alpha + Z_\beta)^2$] to be 234. It was assumed that 80% of all smear positive sputum samples will yield strains on culture. Therefore a total of 292 patients were considered as the required sample size for the study. Sample size calculation was done using EPI-Info statistical software package for cross-sectional/cohort study. Patients were enrolled consecutively until the required sample size was reached.

Where: N= Minimum sample size; P_1=Prevalence of drug resistance in HIV negative; P_2= Prevalence of drug resistance in HIV positive; Z_α= 1.96 and Z_β=power

5.5. Inclusion Criteria and Exclusion Criteria

Inclusion criteria were age greater than or equal to 18 years, the individual willing to participate in the study and sign informed consent for HIV testing. Exclusion criteria were patients with severe illness unable to provide sputum sample.

5.6. Sample Collection

Samples were collected consecutively from smear positive pulmonary TB patients enrolled in the study. For logistic reasons, samples were collected from the five diagnostic and treatment centers close to Shashemene town (Shashemene General Hospital, Shashemene Health Center, Arsi-Negelle Health Center, Aje Health Center and Wondo Genet Health Center), based on their geographical proximity. Samples were collected continuously until the required sample size was achieved. Samples were collected over a period of 8 months at all sites. Every patient was requested to give sputum sample and venous blood for HIV testing and then the three sputum samples (the same samples as given for routine diagnosis) pooled in 20 ml sterile screw capped universal test tubes. Serum was separated from the whole blood and was collected in sterile cryotubes (Nunc, Denmark). Sputum and serum were collected for maximum of four days in diagnostic centers and transported in a cold chain (at +4^{0}C) within one day from the diagnostic centers to the core laboratory at AHRI.

The sputum was then cultured for mycobacteria immediately the next day after transported to AHRI and serum was screened for HIV status and the result was submitted to the respective health center.

5.7. Previous anti-TB treatment status of enrolled patients

Patients were interviewed by nurses from TB center to determine history of previous treatment with anti-TB drugs and to collect information on general socio-demographic factors. To do this a standard WHO questionnaire (WHO, 2003), translated into Amharic was used after pre-testing. Data were verified by referring to patient records obtained from the archives of the health centers where patients had been evaluated for TB treatment. This was intended to confirm previous anti-TB treatment status described by the patient. The quality of the data given was checked by re-interviewing every twentieth patient in the center by another health worker. Additionally, patients subsequently diagnosed to harbor any type of drug resistant *mycobacterial* isolate were re-interviewed by the principal investigator or senior nurse.

5.8. Preparation of Lowenstein Jensen (LJ) Media

Glycerol and pyruvate containing LJ media were prepared. Potassium hydrogen- phosphate, magnesium sulphate, magnesium citrate and asparagin (Sigma, St. Louis, USA) (3 g, 0.3 g, 0.75 g and 4.5 g respectively) were dissolved in 750 ml distilled water at 100^0C. The solution was kept at 4^0C overnight. Malachite green, 1 g, was dissolved in 100 ml distilled water at 56 ^{0}C in water bath. Potato starch (NMD, Bergen) (37.5g) was dry sterilized at 140^0C for 15 minutes and mixed with the solution which was then kept at 100^0C with occasional shaking until it became thick. A well-shaken egg solution (1,750 ml) was then added to the thick solution. This was followed by vigorous shaking of the whole solution.

The mineral salt and egg homogenate-containing solution was then divided into two equal parts. To one of these, glycerol (12.5 ml per 1,250 ml of the solution) was added (glycerol medium) while pyruvate (5 g in 1250 ml of the solution) was added to the other part. The final solutions were then dispensed in 4 ml volumes into culture test tubes which were slanted at 15-20 ^{0} angle and heated at 85 ^{0}C for 55 minutes using an inspissator (TB-GD1001N).

5.9. Mycobacterial culture and isolation of strains

Sputum was digested and decontaminated using Petroff's method (Canetti *et al*,. 1996). Equal amount of 4% NaOH was added to the sputum. Then it was vortexed in a closed tube for 1-3 minutes until the sputum and NaOH mixture became homogeneous. This was followed by centrifugation of the solution at 1,620g for 15 minutes. Most part of the supernatant was discarded and the pellet was resuspended in the remaining supernatant (approximately 500 µl). Phenol red was used as an indicator and HCl for neutralization. Blood streaked sputa were washed with 20 ml PBS after centrifugation rather than neutralization with HCl. Three ml of PBS was added to the neutralized solution and then inoculated on two LJ media, one containing glycerol and the other pyruvate. The remaining 500µl solution was further diluted with 3 ml PBS and inoculated again on to LJ containing glycerol. Culture tubes were incubated at 37^0C and growth was monitored every week. Growth was usually observed at around three weeks. If there was no growth at 8 weeks of incubation, samples were labeled as culture negative and safely disposed off. All culture work was conducted in a biosafety level (P3) TB laboratory at AHRI with all safety precautions in place.

5.10. Preparation of Drug and TCH solutions

Stock solutions of RMP, INH, STM, EMB and TCH (Thiophene-2-carboxylic acid hydrazide) (Sigma, St. Louis, USA) were prepared in phosphate buffered saline (PBS). After filter sterilization using 0.2µM filter, aliquots of the drug solutions were kept in cryotubes (Nunc, Denmark) and stored at -70^oC (Table 5.1). When required, these stock solutions were thawed and added to the 7H10 medium to get the required critical concentration.

Twenty milligram of Isoniazid was dissolved in 100 ml of PBS to get a drug concentration of 0.2 mg/ml (stock solution). This concentration was added to Middlebrook 7H10 media with Middlebrook OADC enrichment to get a final concentration of 0.2 mg/L (Table 5.1.). Twenty milligram of Rifampicin was first dissolved in 2 ml of concentrated ethanol. Adding 18ml of PBS then diluted the drug solution to 1mg/ml. This solution was then added into the medium to get a final concentration of 1mg/L (Table 5.1.). Streptomycin was prepared by dissolving 200 mg of the drug in 80 ml PBS. This drug solution with a concentration of

2.5 mg/ml was added to the medium to get a final concentration of 2 mg/L. Similarly, Ethambutol solution was prepared by dissolving 200mg of the drug in 32 ml PBS. This drug solution with a concentration of 6.25mg/ml was added to the medium to obtain a final concentration of 5mg/L.

Table 5.1. *Source, concentration and solvents of anti-tuberculosis drugs and a chemical used for biochemical test in the study.*

Drugs	**Product Number (Sigma)**	**Solvent(s) used**	**Concentration of drugs**
Rifampicin	R-3501	Ethanol/ PBS	1mg/L
Isoniazid	I-3377	PBS	0.2mg/L
Streptomycin	S-6501	PBS	2mg/L
Ethambutol	E-4630	PBS	5mg/L
TCH	T-1388	PBS	2mg/L

5.11. Preparation of drug containing and drug free Middlebrook 7H10

Middlebrook 7H10 agar (Becton Dickinson, France) was mixed with distilled water (in a proportion of 19 g to 900 ml) with magnetic stirrer at a temperature of 50-60^{0}C. Then (5 ml) glycerol (Sigma, St. Louis, USA) was added to the agar and autoclaved at 120^{0}C for 10 minutes. The media was allowed to cool down to a temperature of 50 to 56^{0}C, 100ml OADC (Becton Dickinson, USA) was then added to make up to 10% of the total constituents (vol/vol) and mixed well. Drugs were added to the media (for the drug-containing media) when the temperature reached 50-52 ^{0}C. In the controls, no drug was added. Then, 4 ml of the media was dispensed into test tubes, which were slanted at around 15-20 degrees for the medium to solidify in that position. The media was then stored at 4^{0}C in the dark.

5.12. Drug Sensitivity determination of Mycobacterial Isolates

Standard indirect drug susceptibility tests were done for Isoniazid, Rifampicin, Streptomycin and Ethambutol. The test was done based on the proportion method (Canetti *et al.*, 1969) using Middlebrook 7H10 supplemented with glycerol and 10% OADC (Oleic Acid Albumin Dextrose Catalase) Briefly, one loop full growth of *Mycobacterium* from a primary culture

was taken and vortexed in a test tube that contained 7-10 glass beads and a drop of PBS. The contents were shaken until it became homogenized. Then, two drops of PBS were added and the suspension vortexed again. Three milliliters of PBS were added and allowed to settle for 30 minutes so that the homogenized suspension could be taken easily and the beads and debris are left in the tube. The bacterial suspension was adjusted to a McFarland turbidity standard-1 (OD value between 0.25 and 0.30 at 600 nm using Novaspec II photometer, Pharmacia Biotech Ltd, UK) with PBS. This concentration was diluted to 1:10 and 1:1,000 (Figure 5.2). From the 1:10 bacterial dilution, 100µl was transferred into all drug-containing media and one drug-free control medium. About 100µl of bacterial suspension from the 1:1000 dilution was transferred into one drug-free control tube (Figure 5.3). All the samples were then incubated at 37^0C and examined for colony formation every week until the third week. Three reference strains (ATCC 35835, ATCC35836 and ATCC35838 which are Isoniazid, Streptomycin and Rifampicin resistant respectively) were included in each test batch as positive and one PBS inoculated tube as negative controls.

Usually, the sensitivity result of the isolate was read by visual comparison of the drug-containing media (on which we inoculated 1:10 bacterial suspension) with the drug free control on which 1:1,000 bacterial suspension was inoculated. The two concentrations of bacterial suspension (i.e. 1:10 and 1:1,000) inoculated on the two media have a factor of 100 between them. Equal growth on the two media, in principle, would mean 1% of the isolates are resistant to that particular drug. However**,** for reason of accuracy, the test was repeated if the growth is equal or nearly equal for both.

The proportion of growth could also be calculated by dividing the number of colonies on a drug-containing medium with the number on the corresponding drug-free control when the numbers of colonies on drug-free media fall between 50 and 150. A bacterial growth of more than 1% was taken as resistant. The proportion of bacteria less than 1% was considered as susceptible.

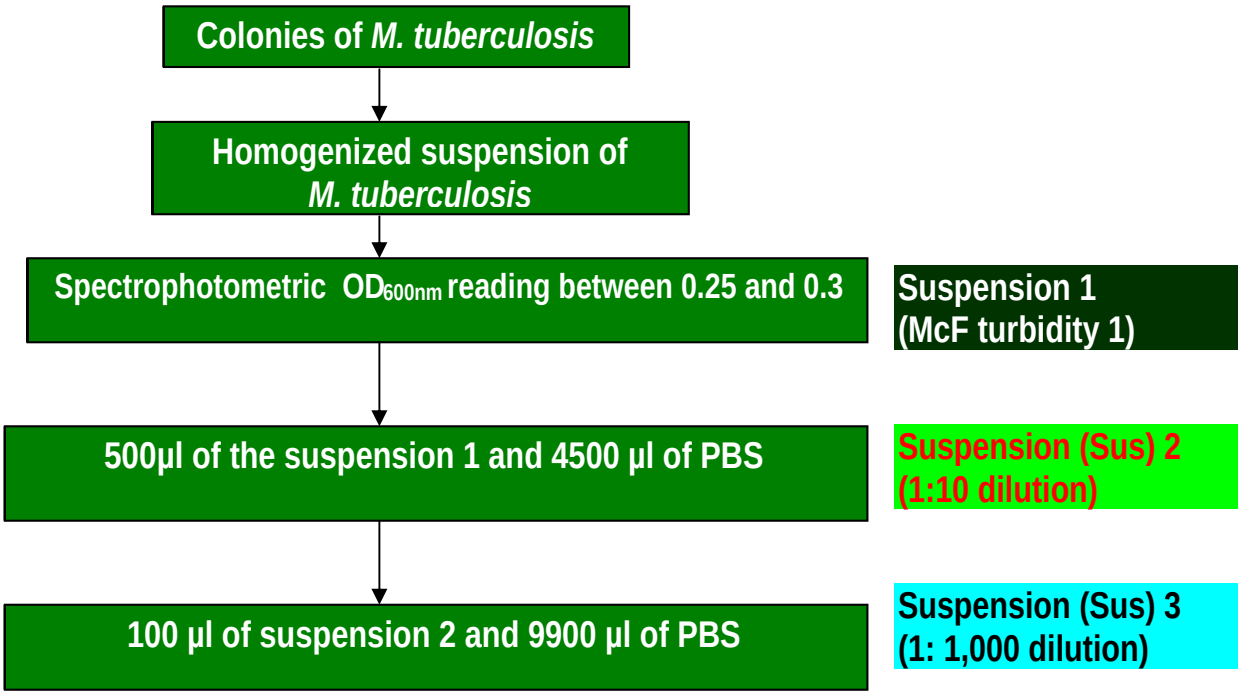
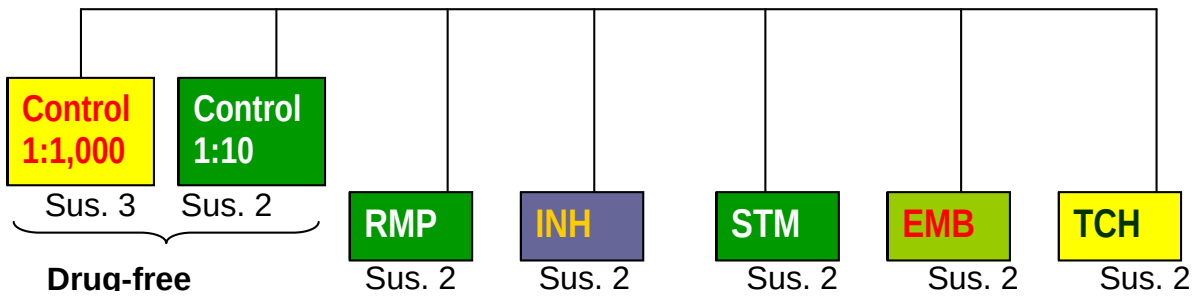


Figure 5.2. *Preparation of different concentrations of mycobacterial suspension from colonies on culture medium*

Figure 5.3. *Reading anti- TB drug susceptibility results.*



Resistant *= Number of colonies on drug-containing media are greater than the number of colonies from corresponding control (Sus.3)*

Sensitive*= Number of colonies on drug-containing media are fewer than the number of colonies from corresponding control (Sus.3)*

5.13. Species identification

Combination of phenotypic and genotypic means of identifying species of *Mycobacterial* isolates was done by using TCH biochemical test (it follows the same procedure like drug sensitivity test) and PCR molecular characterization. The phenotypic test done in this study was Thiophene-2-carboxylic acid hydrazide (TCH) sensitivity determination. The TCH test was used for screening purposes. Isolates sensitive to TCH and randomly selected isolates resistant to TCH and INH were then subjected to genotypic method of species identification (Kent and Kubica, 1985)**.**

5.13.1. Phenotypic method of species identification

Thiophene-2-carboxylic acid hydrazide (TCH) test was used to determine phenotypic species identification between *M. tuberculosis* and *M. bovis*. *M. bovis* is susceptible to low concentration (1 to 5μg/ml) of TCH where as *M. tuberculosis* and other mycobacteria are resistant to the inhibitory action of this compound (Vestal and Kubica, 1967). For each isolate, three tubes of OADC enriched Middlebrook 7H10 medium were prepared where one tube was for TCH and two tubes were for non TCH medium. Filter sterilized TCH solution was added in one of the media to make a final concentration of 2 mg/L. On the drug-containing and drug free media 100μl of 1:10 bacterial suspension (compared to McFarland turbidity standard-1, section 5.12.) was inoculated on the drug containing medium while 100μl of 1:1,000 bacterial suspension was inoculated on the other drug free medium. Then, the media were incubated for three weeks. *M. bovis* strain was used as a positive control in every batch of media prepared. An isolate was considered resistant when growth on the TCH containing medium was equal to or greater than 1% of that observed on the drug-free medium (Similar to the procedure under 5.12).

5.13.2. Genotypic method of species identification

5.13.2.1. Genomic DNA Extraction

The DNA extraction of mycobacteria was performed according to the extraction protocol described by van Soolingen *et al.*, 1995. Bacteria grown on Lowenstein–Jensen (LJ) media were resuspended in 400 μl of 1x TE buffer pH 8.0. The mixture was heated in a water bath at 80 ^{o}C for one hour to kill the bacteria and cooled to room temperature.

Fifty µl of lysozyme (10mg/ml) [Sigma, Saint Louis, USA] was added to lyse the bacteria and the mixture was incubated in a 37^0C water bath for one hour. In the lysosome treated samples, 75µl of 10% SDS/proteinase K mix (Sigma Chemical Corporation) was used and incubation continued for 10 minutes at 65^0C. To remove inhibitors, 100µl of 5M NaCl and 100 µl of cetyltrimethylammonium bromide (CTAB)/NaCl solution were added to the sample (total volume of 725 µl), vortexed and incubated for 10 minutes at 65^0C.

The DNA was extraction by adding an equal volume of chloroform-isoamyl alcohol in 24:1 (volume/ volume) ratio. Four hundred fifty µl of isopropanol was added to the aqueous phase and DNA was precipitate at -20^0C for 30 minutes. The DNA was recovered by centrifugation at 12,000g for 15 minutes. The DNA pellet was washed with 1ml cold 70% ethanol to remove CTAB and NaCl. The ethanol washed pellet was treated with 10 mg/ml DNase free RNase A (Sigma Chemical Corporation) and incubated for one hour at 37^0 C. Finally, the pellet was dried at room temperature, re- dissolved in 1x TE buffer (pH 8.0) at a concentration of DNA (0.1µg/µl -1µg/µl) and stored at 4^0C for immediate processing or -20^0C until required for further analysis.

5.13.2.2. Polymerase Chain Reaction (PCR)

The RD4 region originally described as being deleted in the genomes of BCG isolates relative to the sequence of *M. tuberculosis* H37Rv (Behr, 1999). The genomes of the isolates were analyzed by PCR for the presence or the absence of deleted region. A multi-primer PCR assay with three primers was used to detect RD4 deletion region. The primer sequences for RD4 flanking primer (5'-CTC-GTC-GAA-GGC-CAC-TAA-AG-3'), (5'-AAG-GCG-AAC-AGA-TTC-AGC-AT-3') RD4 Internal primer (5'-ACA-CGC-TGG-CAA-GTA-TAG-C-3'). Primers were selected by the sequences into the Primer 3 program at www.genome.wi.mit.edu.

PCR Mix:

Twenty five µl reaction mixture containing each of the two flanking primers at a concentration of 10µg/µl, 10µg/µl internal primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate (dNTP) at a concentration of 200 µM, 1.25 U of *Taq* DNA polymerase and 0.1 µg of DNA from bacterial cells.

PCR cyclic conditions: initial denaturation at 95°C for 5 min, 40 cycles at 94°C for 30 sec, 1 min for 65°C and 10 min at 72°C, in a MBS 0.25 (Thermo Hybraid) thermocycler.

For electorphoresis, 10µl (7µl PCR product and 3µl of 6x concentrated dye mix) samples were run at 100 V for 1 h on a 1.5% agarose gel, and the band size was estimated by comparison to a 1-kb-plus (1µg/µl) DNA ladder (Gibco BRL, Life Technologies, and Gaithersburg, Md.).

5.14. Polymerase Chain Reaction (PCR) based Analysis of *M. tuberculosis* Mutants

5.14.1. KatG315 Multiplex Allele-Specific (MAS) PCR assay for Isoniazid Resistance

DNA used for the PCR analysis was extracted from *M. tuberculosis* culture and purified as described by van Embden *et al*., 1993.

The multiplex allele-specific (MAS)-PCR assay was previously developed for *embB306* mutational analysis **(**Mokrousov *et al*., 2002**)**. We applied the same strategy to distinguish between the wild-type and mutated alleles of *Kat*G315. The assay used three primers (katg0F – GCA GAT GGG GCT GAT CTA CG, katg4R – AAC GGG TCC GGG ATG GTG and katg5R – ATA CGA CCT CGA TGC CGC). The inner reverse primer katg5R positioned so that its 3- end paired with the second base (G) of the codon 315 wild-type allele (AGC). Consequently, in the absence of mutation in this position in *kat*G315, a 292-bp fragment amplified by the outer forward primer katg0F and the inner reverse primer katg5R (Figure 5.4). If a mutation (e.g., AGC→ACC) occurred, this resulted in a mismatch at the 3'-end of this wild-type inner primer and, under the appropriate stringent PCR conditions, the 292-bp PCR product will not been detected (Figure 5.4). In the case of the *kat*G315 wild-type allele, amplification of this 435-bp fragment was prevented under selected reaction conditions due to the concurrent action of the katg5R inner primer. The 435-bp fragment was amplified in only the strains with *kat*G315 mutations and was therefore indicative of the INH resistance phenotype.

PCR Mix:

DNA sample (0.1μg) or 1μg from culture was added to a PCR mixture (final volume of 30μl) that contained 30 pmol each of katg0F and katg5R, 40 pmol of katg4R, 1.5 mM $MgCl_2$, 1U of recombinant *Taq* DNA polymerase (MBI Fermentas), and 200μM concentrations of each deoxynucleoside triphosphate.

PCR conditions: initial denaturation at 96°C for 3 min; 5 cycles at 95°C for 1 min, 64°C for 1 min, and 72°C for 30 s; 5 cycles at 95°C for 1 min, 62°C for 40 s, and 72°C for 30 s; 20 cycles (25 cycles for sputum) at 94°C for 1 min, 60°C for 40 s, and 72°C for 30 s; and final elongation at 72°C for 3 min. The amplified fragments will be electrophoresed in 1.5% agarose gels and visualized under UV light.

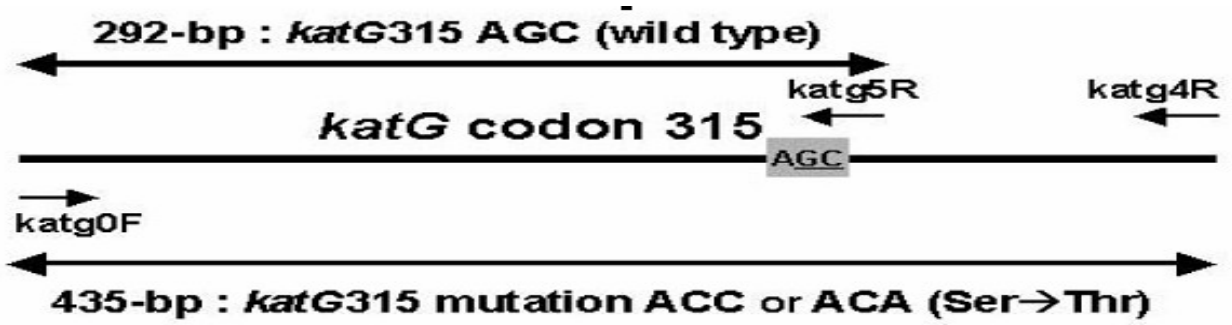


Figure 5.4*. MAS-PCR assay to detect variation in katG315 in M. tuberculosis and schematic view of the katG gene fragment targeted by MAS-PCR assay using three primers (Katg0F, katg5R, katg4R) (Mokrousov et al., 2002).*

5.14. 2. rpsL43 PCR-RFLP assay for streptomycin [STM] resistance

PCR- RFLP assay will be carried out for *rps*L43 mutational analysis. In this study, we applied the same strategy like Mokrousov *et al*., 2004, to distinguish between the wild-type and mutated alleles of *rps*L43 using PCR-RFLP. The assay used two primers (RPSF78- 5'-CAG CCC GCA GCG TCG TGG TG and RPSR322- 5'-GCT GCG TGC CTG TTT GCG GTT CTT). In the absence of mutation in *rpsL*43 region, *Mbo*II restriction enzyme digests *rps*L43 and results a 210-bp and 60-bp fragment. If mutation will be occurred in *rps*L codon 43 (AAG→AGG) PCR fragment remains intact after *Mbo*II digestion and this will be resulted in a 270-bp.

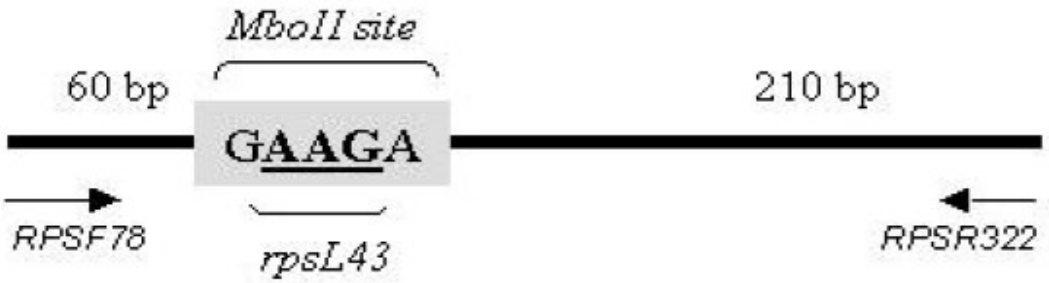


Figure 5.5. *PCR-RFLP detection of rpsL43 AAG>AGG mutation in M. tuberculosis and schematic presentation of the rpsL43 fragment (Mokrousov et al., 2004).*

PCR Mix:

DNA sample (0.1μg or 1μg) added to a PCR mixture (final volume of 30μl) that contained 20 pmol each of RPSF78 and RPSR322, 1.5 mM MgCl2, 1U of recombinant *Taq* DNA polymerase, and 200 μM concentrations of each deoxynucleoside triphosphate.

PCR cyclic conditions: initial denaturation at 96°C for 3 min; 2 cycles at 96°C for 20s, 65°C for 1 min, and 72°C for 20 s; 2 cycles at 96°C for 20s, 63°C for 1min, and 72°C for 1min; 2 cycles at 96°C for 20s, 61°C for 1min, and 72°C for 1min; 2 cycles at 96°C for 20s, 59°C for 1min, and 72°C for 1min; 2 cycles at 96°C for 20s, 57°C for 1min, and 72°C for 1min; 20 cycles (25 cycles for sputum) at 96°C for 20s, 55°C for 1min, and 72°C for 1min; and final elongation at 72°C for 5 min. The amplified fragments were 5 μl loaded in 1.5% agarose mini-gel.

***Mbo*II-RFLP**

MboII restriction enzyme digest amplified *rpsL43* fragment with 1-2 units of *Mbo*II, in final volume 10 μl. For one sample, it was used 7 μl PCR product, 1 μl 10x buffer and 2μl *Mbo*II (1 unit) and tubes was closed, mix, spin, and incubated for 2-3 hours at 37 ºC.

5.16. HIV Testing

Five ml of venous blood was drawn aseptically from each patient and serum was separated by centrifugation in the Health Centers and the Hospital. The serum was transferred into a cryotube (Nunc, Denmark) and stored at -20^{0}C until it was transported in a cold chain to AHRI laboratory.

The sample was first tested with Determine® (ABOTT JAPAN CO., Tokyo), a rapid test with higher sensitivity. Positive samples were again confirmed with Capillus® (Trinity Biotech, Ireland), a rapid test with higher specificity. If it is positive in assay one turned out to be negative in the confirmatory test, assay one would be repeated in order to rule out any possible technical errors. In this case if the test result of the repeated first assay is positive, the sample is reported as positive. If there was still a discordant result, the sample would be tested with a third kit, Unigold® (Trinity Biotech, Ireland), as a tie-breaker. The result of the tie-breaker test will be the accepted final result (*National Guidelines for Voluntary HIV Counseling and Testing in Ethiopia,* MoH, 2004).

5.17. Quality Control

The quality of the LJ medium to grow *Mycobacteria* was checked by inoculating media with *Mycobacterium gordoneae*, a rapidly growing species. Growth of this organism was checked for 3-4 days. The color of the media was checked before using it for culture and only those media with appropriate color (light green) were chosen. Media with a color between deep green to blue were frequently observed to give negative cultures with further color changes during incubation. This was believed to be due to the acidic pH of the media. Therefore careful adjustment of the inoculum pH (7.2- 7.4) was mandatory to avoid overwhelming the already diminished buffering capacity of the media.

Internal quality control strains with a known drug sensitivity pattern (ATCC35835, ATCC35836 and ATCC35838 were standard strains for INH, STM and RMP resistance respectively) were processed at the same time as samples were processed, and at every run. Only when the expected result of the internal quality control strain was achieved were the

tests considered valid. The internal quality control strain concordance rate was 100% with the expected drugs.

5.18. Ethical Consideration

Ethical approval was obtained from Addis Ababa University, AHRI/ALERT Ethics Committee, the National Ethical Review Committee and support letter from the Regional Health Bureau. Patients were informed about the study using an approved study protocol. Informed consent was obtained from all study participants. HIV test results were provided through the health facility counselor. Confidentiality was assured through use of codes in records.

5.19. Statistical Analysis

Socio-demographic and clinical data obtained through questionnaires and the results of laboratory tests were entered into EPI-Info (Version 2004) (CDC, WHO) data record files. Statistical analysis was performed using EPI-Info (Version 2004), STATA and SPSS software packages (version 13.0). The Chi-square (X^2) test was used to detect statistically significant differences. Adjusted analysis of proportions was made by logistic regression. The odds ratio was used to measure degree of association. A probability of <0.05 was considered significant.

6. RESULTS

This is the first report for such type of study in the Shashemene area of Southeast Ethiopia. It was a health facility based survey and was conducted in the Shashemene area of Southeast of the country. The study covered both molecular and conventional epidemiology of drug resistance of *Mycobacterial* isolates among HIV positive and HIV negative TB patients. Samples were collected, by considering the representativeness of the sample from health centers and the hospital as well as both the road sided urban and rural centers.

6.1. Sociodemographic characteristics and Drug Susceptibility

Sputum and serum were collected from 292 patients' consecutively who agreed to participate in the study at the study sites (Shashemene Hospital, Wondo Genet Health Centre, Shashemene Health Centre, Arsi-Negelle Health Centre and Aje Health Centre). The data presented here are based on the 241 patients for whom isolates were tested for drug susceptibility. No previous treatment was reported for 265 of the patients. All samples were cultured and out of this 241 (82.50%) were culture positive. The rest were either culture negative (n= 45 [15.4%]) or contaminated (n= 6 [2.1%]). It is suspected that most (>80%) of the negative cultures were due to technical failures (difficulty of adjusting pH of the inoculum). Anti-TB drug sensitivity testing was done on 241 isolates from 217 newly diagnosed and 24 previously treated pulmonary TB patients (Figure 6.2.).

The study participants comprised of 51.5 % (124) males and 48.5% (117) females. The age range of the patients was between 18 and 65 years and mean age of 27.1 ($\pm$10.13) years (Figure 6.1). The mean body mass index (BMI) of the patients ranged between 11.7 and 26.3 and a mean BMI of 17.6 ($\pm$4.3). Among the study participants, 45.2% were single, 48.5% married, 3.7% divorced and 2.5% others. The proportion of students was 30.7 % where as housewives, farmers, unemployed and others comprised 28.2%, 19.9%, 10.0% and 11.2% of the study participants respectively (Table 6.1).

Fever was reported in 83.0% (200/241) of the study participants. Chest pain was a complaint in 85.1% (205) and hemoptysis in 17.9%. Among those for whom chest X-rays were done

(n=146) pulmonary cavity was observed in 51.8%. No radiologic findings of cavity were apparent in 48.2% despite clinical disease (Table 6.1).

Socio-demographic and the clinical presentations of TB patients were compared for culture positive and culture negative patients. There was no statistically significant difference between the two groups for the variables compared.

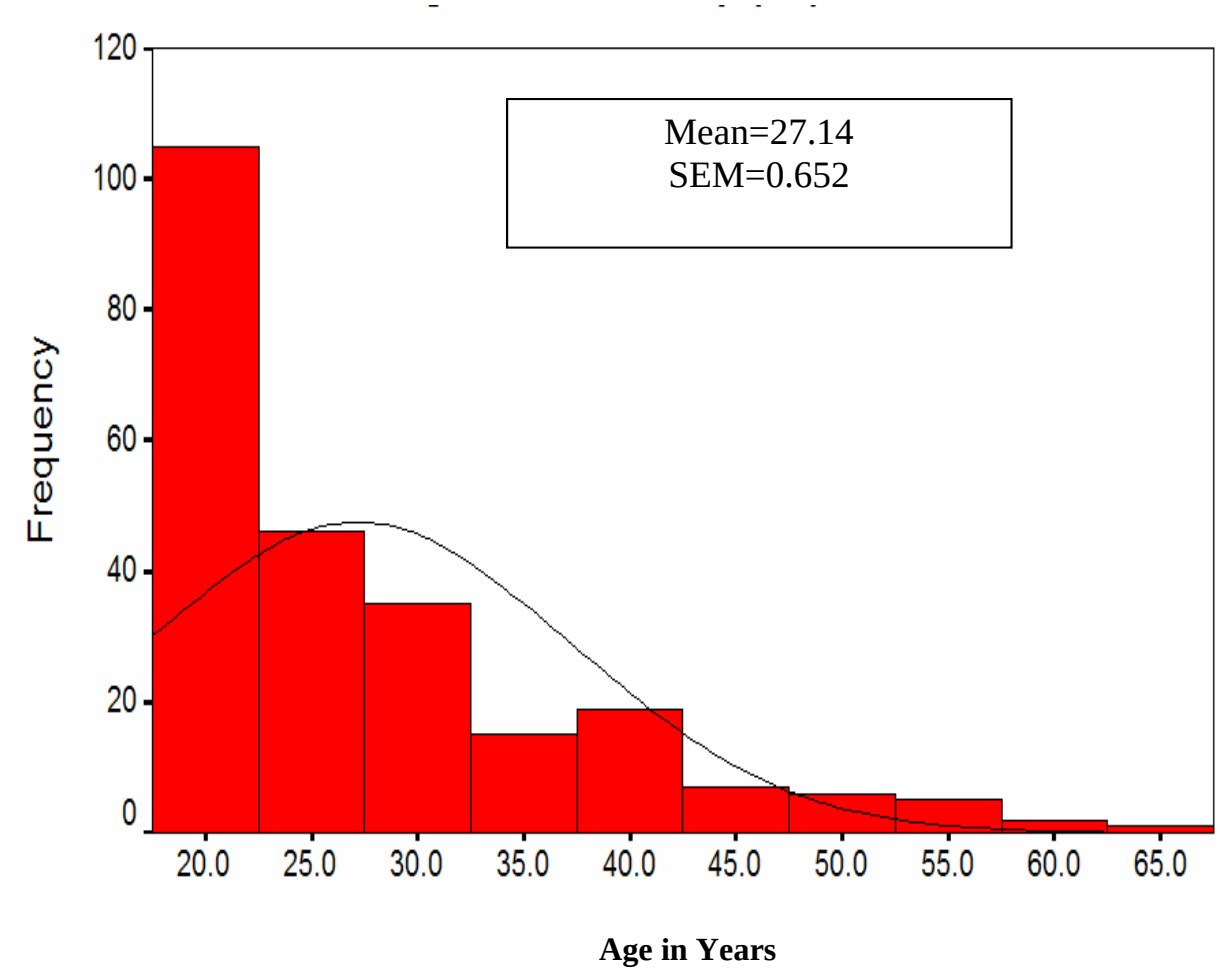


Figure 6.1. *Age distribution of the study population.*

Any type of alcohol consumption was reported by 18.2% (44) of the study population while cigarette smoking was reported in 11.2% (27). History of imprisonment was given by 7.5% while 4.2% of the patients had previously been admitted to hospital (Table 6.1).

Among the newly diagnosed TB cases, resistance to at least to one drug was 19.8% (n=43). i.e. 80.2% (n=174) of the isolates were susceptible to all four anti-TB drugs tested. Any single drug resistance was most frequent to Streptomycin, which was 16.6%. Isoniazid was the second most frequent drug to which any type single drug resistance was observed (12.9%). Single drug resistance to Rifampicin and Ethambutol was 1.8% and 4.1% respectively (Table 6.2).

Combined drug resistance among new TB patients was observed more frequently to INH and STM (7.4%) than to any combination of all others (INH+RIF+STM, INH+STM+EMB, STM+EMB). Triple resistance to INH, STM and EMB was 1.8%. MDR was observed in two isolates (0.9%) from previously untreated patients (Table 6.2).

As expected, resistance was more frequent among previously treated cases than among newly diagnosed TB patients. Resistance to any type of drug was 45.8% among previously treated cases. Among the isolates, 54.2 % were susceptible to all four anti-TB drugs tested. Resistance to Streptomycin, Isoniazid, Ethambutol or Rifampicin was 41.7%, 37.5%, 12.5% and 8.3% respectively (Table 6.2). The most frequent double resistance among previously treated TB patients was to INH and STM (20.8%). Triple resistance to INH, Streptomycin and Ethambutol was observed in 8.3% of the patients. MDR was detected in 8.3% of the isolates from previously treated patients (Table 6.2).

Among the newly diagnosed TB patients with HIV co-infection, the prevalence of any type drug resistance was 18.4% while this figure was 20.2% in HIV negative TB patients. The prevalence of combined resistance to INH and STM was also 10.2% and 11.4% in HIV positive and negative TB patients respectively. These differences were not statistically significant. The prevalence of other types of drug resistance was also not significantly different in HIV positive and HIV negative TB patients (Table 6.4). This absence of significant difference between the two groups was also observed in previously treated patients.

Prevalence of drug resistance was also compared between Shashemene ("Urban" highway town) and "Rural" (smaller off –highway towns) in the study area. There seems to be a

slightly higher prevalence of drug resistance in the "rural" parts. However, all drug resistance parameters were not significantly different in the two locations (Table.6.5). The distribution of any type of drug resistance was also compared among centers from which samples were collected and there was no significant difference observed (ANNEX I).

Resistance to at least to one drug was 45.8% in previously treated patients while this figure was 19.8% among new cases (Table 6.2.). This difference was statistically significant. This association was also seen when multiple drug resistance was compared with previous treatment status (p=0.046). Other socio-demographic factors and behavioral risk factors did not show any significant association with drug resistance.

When we compare each parameter of drug resistance between previously treated cases and new cases of TB, all parameters were higher in the previously treated patients. However among those, statistical difference was not seen for RMP and EMB. Otherwise the difference seen for any type drug resistance (p=0.006), any type of INH resistance (p=0.004), any type of STM resistance (p=0.006), combined type of STM and INH resistance (p=0.004) and multiple drug resistance (p<0.001) were all higher in the previously treated TB patients (Figure 6.3).

The HIV prevalence rate among the total study population (n=241) was 25.7% (62/241). The HIV co-infection rate among newly diagnosed TB patients (n=217), was 22.6% (49/217) in contrast to 54.2% (13/24) among previously treated TB patients (n=24). This difference was statistically significant (p=0.02). This association still existed when it was adjusted for age, sex and occupation. (Table 6.1).

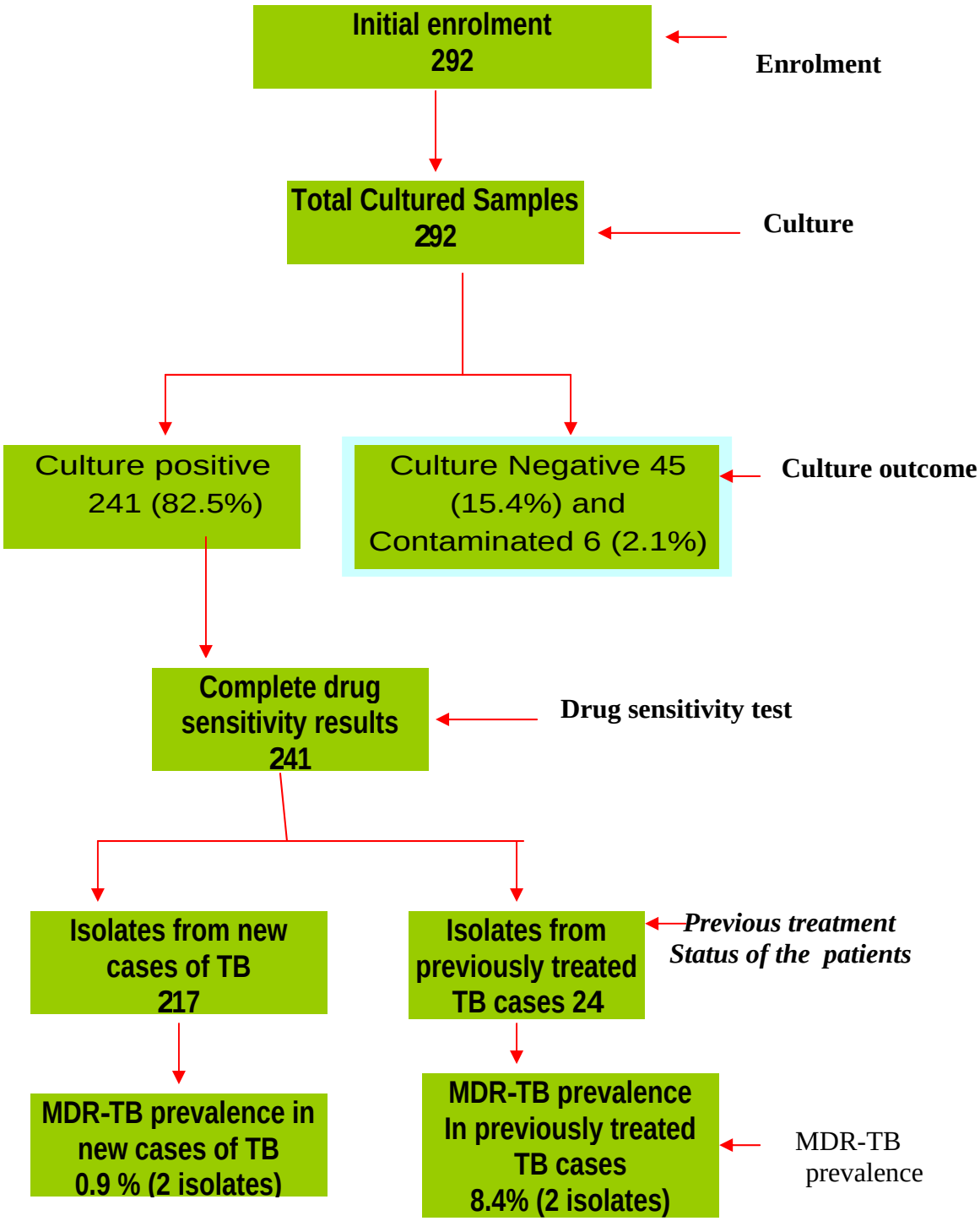


Figure 6.2. Flow chart indicating some of the procedures and outcome of the study.

Table 6.1*. The distribution of the study population by socio-demographic characteristics, behaviour and HIV status, Shashemene area, Southeast Ethiopia, 2006/07.*

Characteristics	New cases (*N=217*) n (%)	Previously treated (*N=24*) N (%)	P value
Age			
18-25	125 (51.9)	12 (5.0)	0.358
26-35	54 (22.4)	8 (3.3)	
36-45	23 (9.5)	3 (1.2)	
46+	16 (6.6)	1 (0.4)	
Gender			
Male	107 (44.4)	17 (7.1)	0.036*
Female	110 (45.6)	7 (2.9)	
Occupation			
Student	70 (29.0)	4 (1.7)	
Housewife	62 (25.7)	6 (2.5)	0.149
Farmer	42 (17.4)	6 (2.5)	
Unemployed	22 (9.1)	2 (0.8)	
Others	21 (8.7)	6 (2.5)	
Hemoptysis			
Yes	38 (15.8)	5 (2.1)	0.402
No	179 (74.3)	19 (7.9)	
Cavity**			
Yes	115 (47.7)	10 (7.5)	0.201
No	102 (42.3)	14 (2.5)	
Ever smoking			
Yes	20 (8.3)	7 (2.9)	0.010*
No	197 (81.7)	17 (7.1)	
Any alcohol intake			
Yes	35 (14.5)	9 (3.7)	0.018*
No	182 (75.5)	15 (6.2)	
Hospitalization***			
Yes	6 (2.5)	4 (1.7)	0.010*
No	211 (87.6)	20 (2.5)	
Imprisonment			
Yes	14 (5.8)	4 (1.7)	0.089
No	203 (84.2)	20 (8.3)	
HIV			
Positive	49 (20.3)	13 (5.4)	0.002*
Negative	168 (69.7)	11 (4.6)	

**Statistical association, **total number =144, ***admission to a hospital for any reason*

Table 6.2. *Pattern of anti-TB drug resistance among newly diagnosed and previously treated TB patients in Shashemene area, Southeast Ethiopia, 2006/07.*

Drug resistance pattern	New cases *n (%)*	Previously treated cases *n (%)*
Sensitive to all drugs	**174 (80.2)**	**13 (54.2)**
Resistant to any one drug	**43 (19.8)**	**11 (45.8)**
Resistance to each drug		
Isoniazid	**28 (12.9)**	**9 (37.5)**
Rifampicin	**4 (1.8)**	**2 (8.3)**
Ethambutol	**9 (4.1)**	**3 (12.5)**
Streptomycin	**36 (16.6)**	**10 (41.7)**
Resistance to more than one drug		
H+R+S + E	**0**	**1 (4.2)**
H+R+S	**2 (0.9)**	**1 (4.2)**
H+S+E	**4 (1.8)**	**2 (8.3)**
H+S	**16 (7.4)**	**5 (20.8)**
S+E	**1 (0.6)**	**0**
Mono resistance		
R alone	**2 (0.9)**	**0**
H alone	**5 (2.3)**	**0**
E alone	**2 (0.9)**	**1 (4.2)**
S alone	**10 (4.6)**	**1 (4.2)**

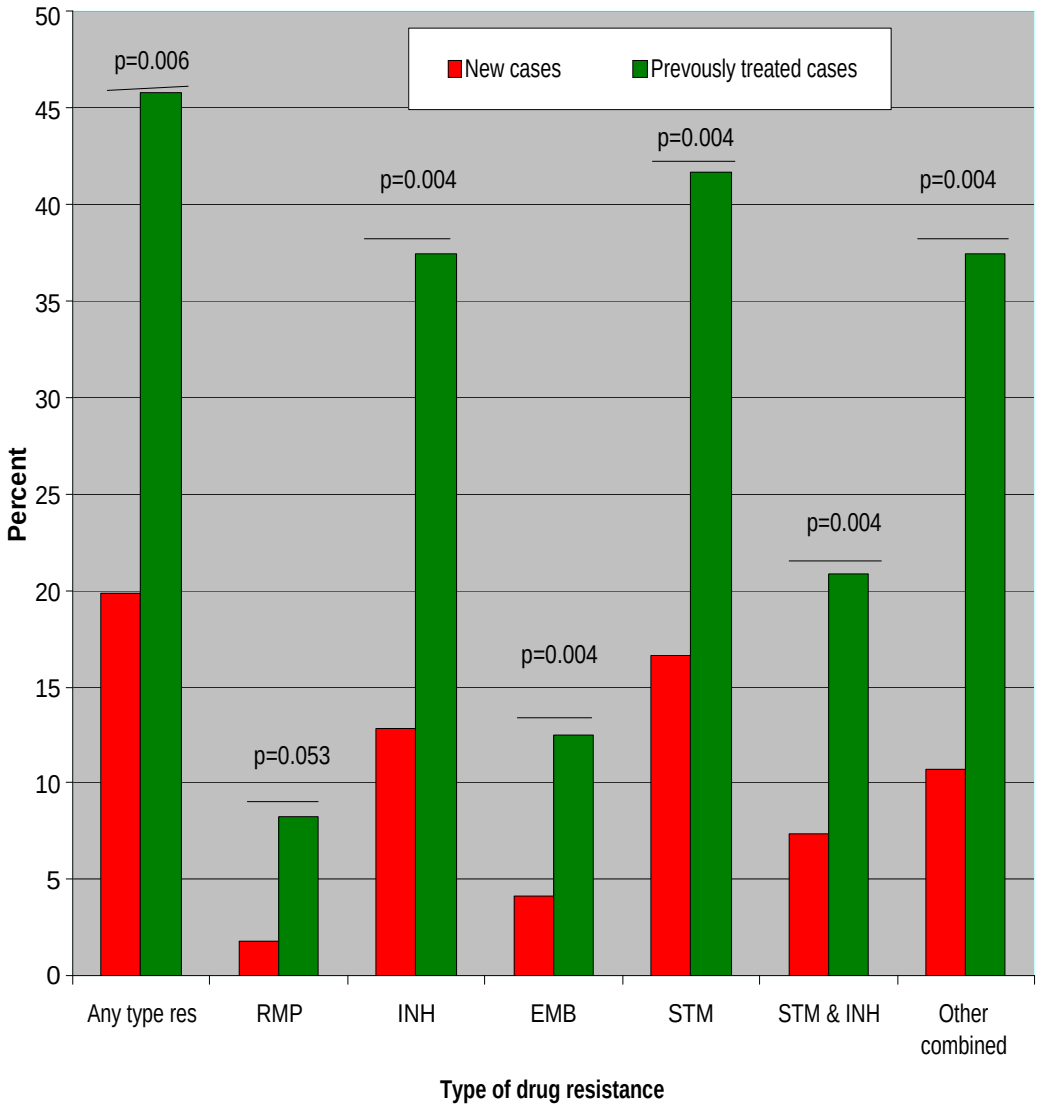


Figure 6.3. *Comparison of prevalence of anti-TB drug resistance pattern among new and previously treated cases, Shashemene area, Southeast Ethiopia, 2006/07.*

Any type res= Resistance to any of the first line anti-TB drugs; RMP= Rifampicin; INH=Isoniazid; EMB=Ethambutol; STM=Streptomycin; INH &STM= Isoniazid and Streptomycin; Multiple res= Resistance to more than one drug.

***Table 6.3**. Association between selected patient characteristics and any type of anti-TB drug resistance among TB patients in Shashemene area, Southeast Ethiopia, 2006/07.*

Characteristics		Resistant n(%)	Sensitive n(%)	OR (95% CI)
1. Previous treatment (n=241)				
	No	43 (17.8)	174 (72.2)	*1.00*
	Yes	11 (4.6)	13 (5.4)	*0.292 (0.12,0.69)**
2. HIV				
	No	34 (15.7)	134 (61.8)	*1.00*
	Yes	9 (4.1)	40 (18.4)	*1.13 (0.50,2.55)*
3. Age				
	40+	8 (3.7)	25 (11.5)	*1.00*
	34-39	12 (5.5)	24 (11.1)	*0.69 (0.27,1.76)*
	18-34	23 (10.6)	125 (57.6)	*1.15 (0.48, 2.76)*
4. Gender				
	Male	24 (11.1)	83 (38.2)	*1.00*
	Female	19 (8.8)	91 (41.9)	*0.72 (0.37,1.41)*
5. History of imprisonment				
	No	40 (18.4)	163 (75.1)	*1.00*
	Yes	3 (1.4)	11 (5.1)	*0.90 (0.24,3.38)*
6. History of hospitalization**				
	No	41 (18.9)	170 (78.3)	*1.00*
	Yes	2 (0.9)	4 (1.8)	*0.48 (0.09,2.72)*
7. Any alcohol consumption				
	No	32 (14.7)	150 (69.1)	*1.00*
	Yes	11 (5.1)	24 (11.1)	*0.465 (0.21, 1.05)*
8. Ever smoking				
	No	37 (17.1)	160 (73.7)	*1.00*
	Yes	6 (2.8)	14 (6.5)	*0.54 (0.19,1.50)*

*Characters 2-8 calculated only for new TB cases; *Statisticaly significant association; **Hospitalization for any illness*

Table 6.4. *Comparison of drug resistance between HIV positive and HIV negative newly diagnosed smear positive TB patients in Shashemene area, Southeast Ethiopia, 2006/07 (n=217)*

	HIV		*P value*
	Positive n (%)	Negative N (%)	
Any type of drug resistance			
Resistant	9 (4.1)	34 (15.7)	0.002*
Sensitive	40 (18.4)	134 (61.8)	
INH			
Resistant	6 (2.8)	22 (10.1)	0.547
Sensitive	43 (19.8)	146 (67.3)	
RMP			
Resistant	0 (0.0)	4 (1.8)	0.358
Sensitive	49 (22.6)	164 (75.6)	
STM			
Resistant	5 (3.7)	28 (12.9)	0.575
Sensitive	41 (18.9)	140 (64.5)	
EMB			
Resistant	1 (0.8)	8 (3.7)	0.357
Sensitive	48 (22.1)	160 (73.7)	
STM+INH			
Resistant	5 (2.3)	18 (8.3)	0.618
Sensitive	44 (20.3)	150 (69.1)	
STM+INH+EMB			
Resistant	1(0.8)	4 (1.8)	0.714
Sensitive	48 (22.1)	164 (75.6)	

Table 6.5. *Comparison of drug resistance among new smear positive TB patients diagnosed at "Urban" centres and "Rural" towns near Shashemene, Southeast Ethiopia, 2006/07 (n=217)*

	Location of the diagnostic center		*P value*
	"Rural" town N (%)	Urban Center N (%)	
Any type of drug resistance			
Resistant	16 (7.4)	27 (12.4)	0.406
Sensitive	59 (27.2)	115 (53.0)	
INH			
Resistant	7 (3.2)	21 (9.7)	0.178
Sensitive	68 (31.3)	121 (55.8)	
RMP			
Resistant	0	4 (1.8)	0.181
Sensitive	75 (34.6)	138 (63.6)	
STM			
Resistant	13 (6.0)	23 (10.6)	0.486
Sensitive	62 (28.6)	119 (54.8)	
EMB			
Resistant	3 (1.4)	6 (2.8)	0.621
Sensitive	72 (33.2)	136 (62.7)	
STM+INH			
Resistant	6 (2.8)	17 (7.8)	0.633
Sensitive	69 (31.8)	125 (57.6)	
STM+INH+EMB			
Resistant	1 (0.46)	4 (1.8)	0.191
Sensitive	74 (34.1)	138 (63.6)	

Most participants in this study 157 (65.1%) lived in urban settings (Table 6.6.). There was strong association between area of residence and HIV infection, with 5.8% (14) of the rural patients and 19.9% (48) of the urban patients being HIV positive (p=0.001) (Table 6.6).

Table 6.6*. Demographic characteristics and disease classification of the study population by HIV status.*

	HIV-positive	HIV-negative	Odds ratio (95% CI)	*pvalue*
	n (%)	*n* (%)		
Gender				
Male	31(20.5)	120 (79.5)	0.775 (0.445- .348)	0.22
Female	34 (25.0)	102 (75.0)		
Residence				
Urban	48 (19.9)	109 (45.2)	0.454 (0.233- 0.885)	0.001*
Rural	14 (5.8)	70 (29.0)		
Treatment history				
New cases	49 (22.6)	168 (77.4)	4.052 (1.708- 9.611)	0.001*
Previously treated	13 (54.2)	11 (45.8)		

The occurrence of drug resistance was not related to either HIV infection status. However, the highest rate of resistance and highest prevalence of HIV infection occurred in the group aged 25- 34 years (Figure 6.4).

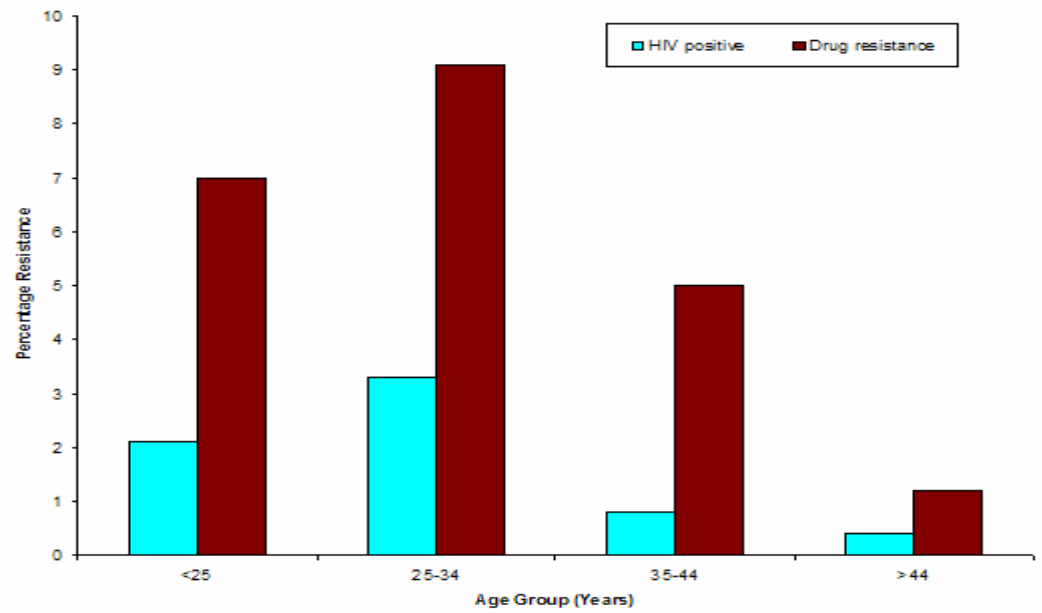


Figure 6.4. *Prevalence of HIV infection and overall drug resistance among Southeast Ethiopian patients with pulmonary TB stratified by age group.*

6.2. SPECIES IDENTIFICATION

Twenty of 241 (8.3%) isolates were sensitive to Thiophene-2-carboxylic acid hydrazide (TCH) possibly *M. bovis* while the remaining 221 isolates were resistant to TCH. Genotypic means of identifying the strain as either *M. tuberculosis* or *M. bovis* was done on all the TCH sensitive strains and on 24 randomly selected strains that were resistant to both INH and TCH. All of these strains were amplified with RD4 primers.

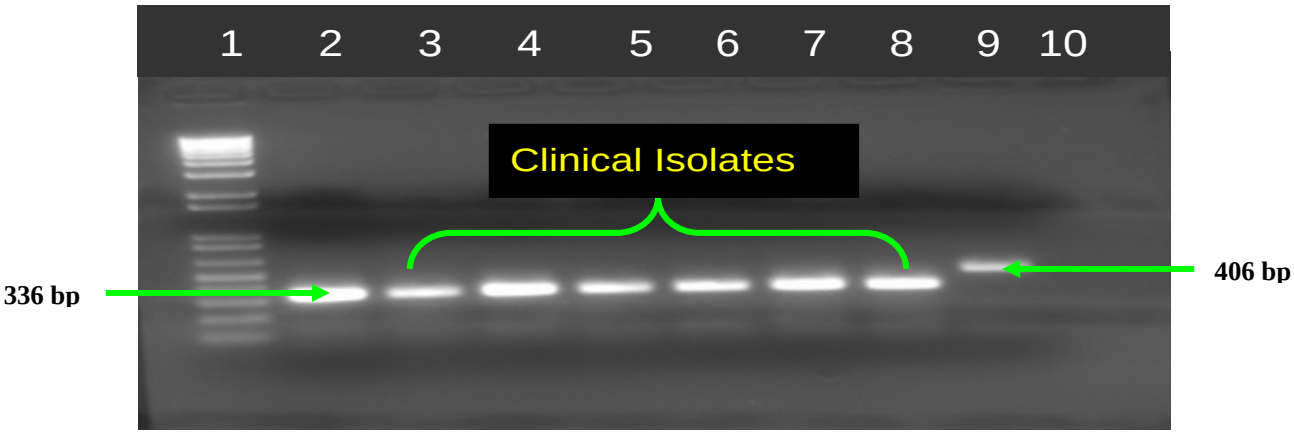


Figure 6.5. *Gel picture of PCR amplification product of culture positive TB strains sensitive to TCH with RD4 primer for species differentiation. L1: Ladder, L2: positive control [M. tuberculosis (H37Rv)] L9: positive control (M. bovis) L3-L8: clinical isolates and L10: negative control.*

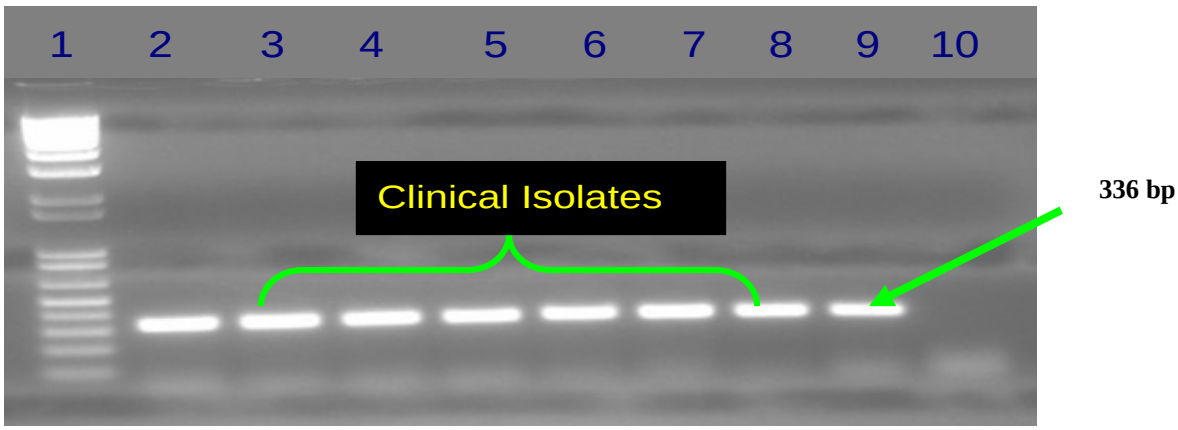


Figure 6.6. *Gel picture of PCR amplification product of culture positive TB strains sensitive to TCH with RD4 primer. L1: Ladder, L2: positive control (*M. tuberculosis*), L3-L9: Culture positive clinical samples, L10: negative control*

6.3. MUTATION ANALYSIS

katG315 Allele-Specific PCR assay for isoniazid [INH] resistance

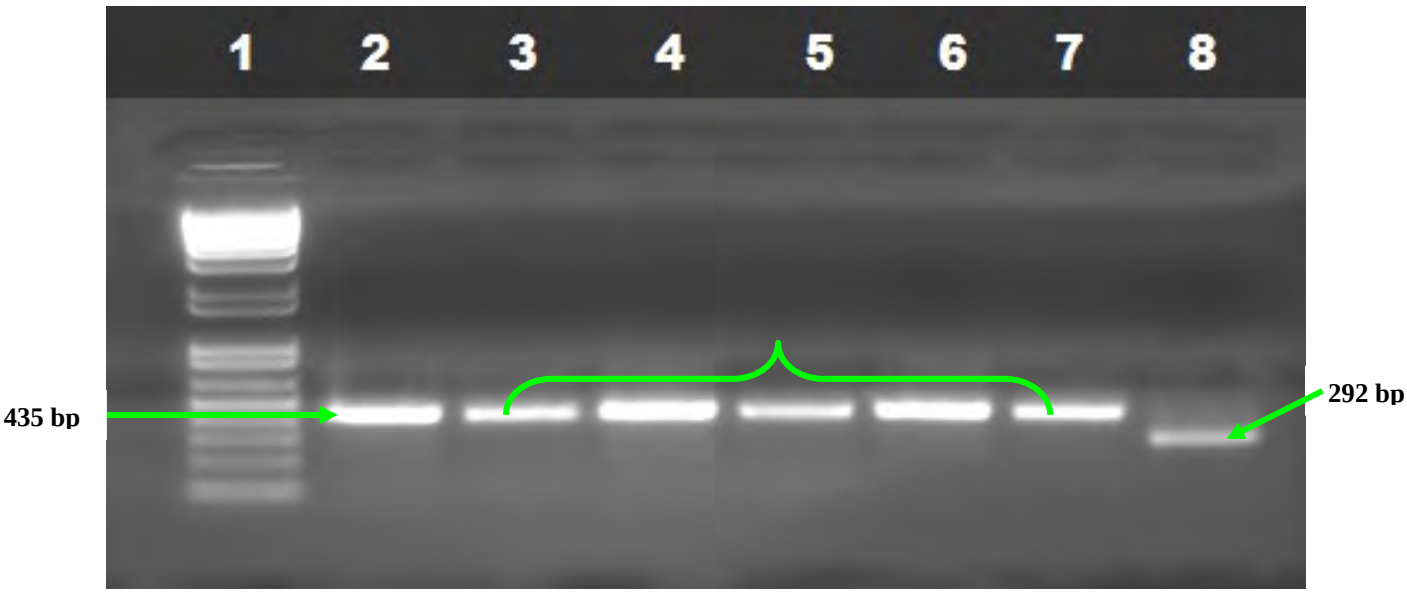


Figure 6.7. *Gel picture of multiplex allele-specific (MAS)-PCR profiles from drug containing media. L1-1kb ladder; L2 mutant (ATCC 35835 reference strain); L3-L7 katG315 mutant alleles and L8- wild type.*

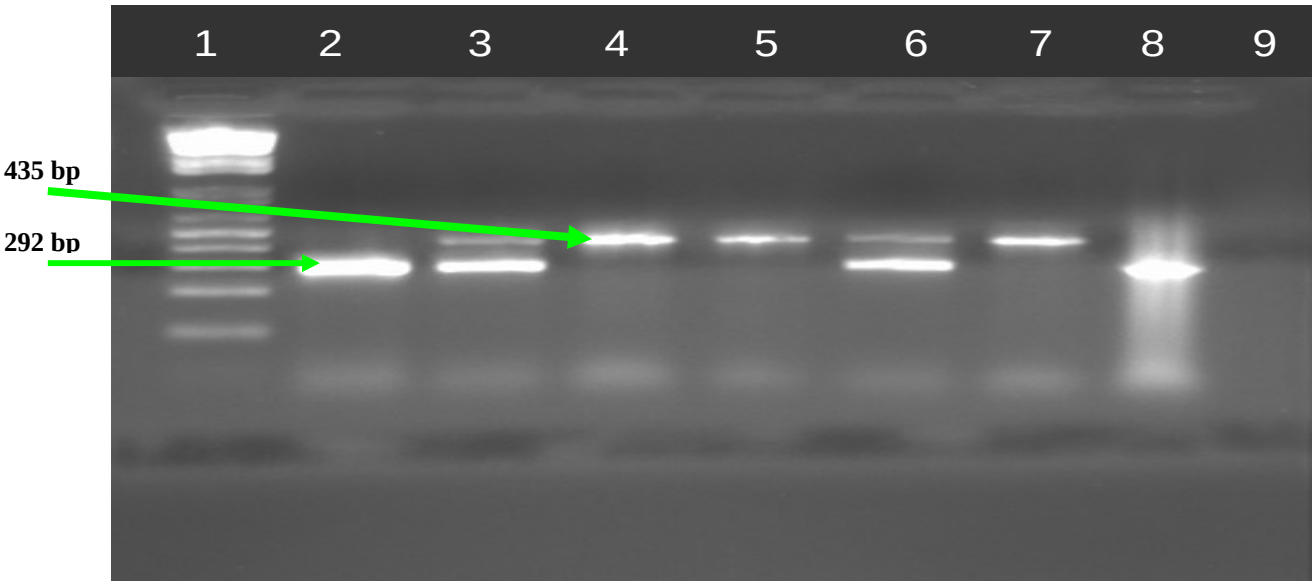


Figure 6.8. *Gel picture of MAS-PCR profiles. L1-1kb ladder; L2 - katG315 wild type (H37Rv); L4- katG315 mutant (ATCC 35835) and L3, L5- L7 were katG315 mutant alleles (INH-resistant strains), L8 wild type and L9–negative control.*

The multiplex allele specific (MAS)-PCR assay was evaluated to detect *katG* codon 315 alteration in the strains studied. All INH susceptible strains produced a single MAS-PCR 292-bp band (Fig. 6.7.), as expected, implying no mutation in the *katG315* second base. A single MAS-PCR 435-bp fragment was amplified in all the 31 out of 37 INH resistant strains harboring the AGC→ACC mutation (Fig. 6.7.). The generation of an either 292- or 435-bp fragment provided a PCR quality confirmation so as to rule out eventual false-negative results due to lack of amplification.

The prevalence of the *katG315* ACC mutant allele among INH resistant *M. tuberculosis* clinical isolates in Shashemene area of Southeast Ethiopia was 83.8% (31/37) for all isolates.

In contrast, the MAS-PCR assay that we used to detect the AGC→ACC mutation. In such a case, a mismatch at the 3'-end of the inner wild-type primer katg5R is even more theatrical, as it involves two bases of codon 315. MAS-PCR was performed on one available ATCC35835 reference strain. This analysis also identified the presence of this mutation and hence the INH resistance phenotype of the ATCC35835 (Fig. 6.7).

The MAS-PCR assay was initially performed on the purified DNA preparations with which the PCR conditions and concentrations were optimized. The assay was then tested on a selection of 20 DNA samples from crude cell lysates and sputum, all from the same patients (five strains, INH susceptible, *katG315* AGC; 15 strains, INH resistant, *katG315* ACC). However, a double band was obtained the from the crude cell lysates and sputum of samples (Fig. 6.8.).

rpsL43 PCR-RFLP assay for streptomycin [STM] resistance

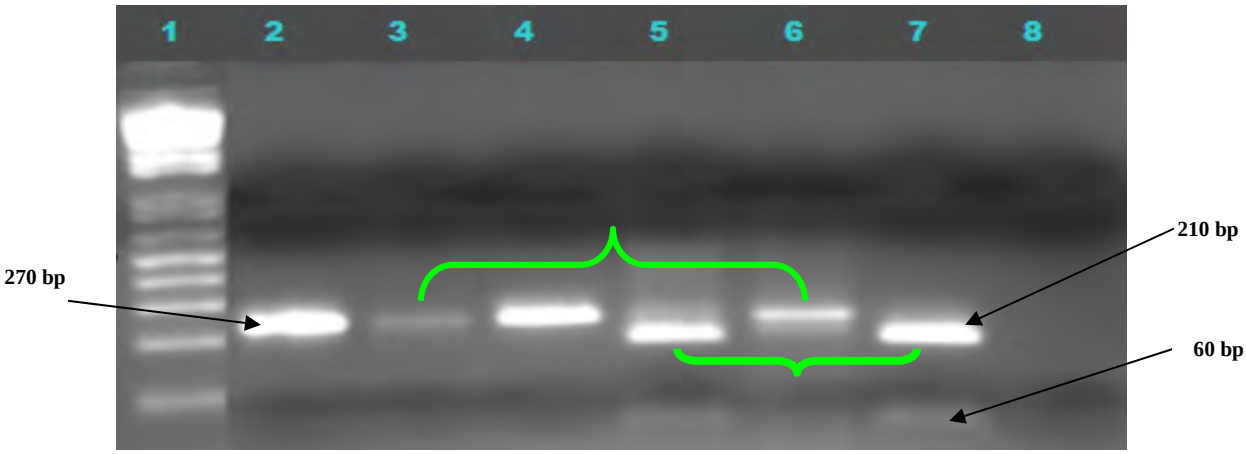


Figure 6.9. *Gel picture of rpsL43 gene PCR-RFLP profiles from drug containing medium. L1-1 kb ladder; L2 mutant (ATCC 35836); L3,L4, and L6 rpsL43 mutant alleles (STM-resistant strains);L5-wild type isolate L7 wild type (control) and L8 negative control.*

The PCR-RFLP assay was evaluated to detect *rpsL* codon 43 (AAG→AGG) alteration in the strains studied. All STM susceptible strains produced 210-bp and 60-bp bands (Fig. 6.9). A single PCR-RFLP 270-bp fragment was amplified in all the 26 out of 36 STM resistant strains harboring the AAG→AGG mutation (Fig. 6.9). Lane 5 isolate was drug resistant in *In-vitro* test but mutation was not occurred in the *rps*L43 gene and produced 210-bp and 60-bp bands (Figure 6.9). Mutation in codon 43 resulted in a 270-bp because *Mbo*II site was lost and the enzyme will not digest the amplified fragment.

The prevalence of the *rpsL*43 AGG mutant allele among the STM resistant *M. tuberculosis* clinical isolates in Shashemene area of Southeast Ethiopia was 55.56% (20/36) for all isolates.

7. DISCUSSION

Tuberculosis is the leading cause of death in hospitalized patients in Shashemene. However, it has not been investigated whether MDR-TB contributes to the reported mortality. There has not been any previous anti-TB drug resistance surveillance in the Shashemene area, Southeast Ethiopia. Most of the studies done were found on the central part of the country, mainly focusing on Addis Ababa city. Thus, present study describes both molecular and conventional epidemiology of drug resistance of *Mycobacterial* isolates among HIV positive and HIV negative TB patients. The study provided the prevalence of (i) any type of anti-TB drug resistance among HIV positive and HIV negative TB patients, (ii) multiple drug resistance among HIV positive and HIV negative TB patients, (iii) multi-drug resistance among HIV positive and HIV negative TB patients, and (iv) single drug resistance to INH, RMP, STM and EMB among HIV positive and HIV negative TB patients. The study also demonstrate the presence of INH and STM drug resistance mutant strains by using *Kat*G315 MAS PCR and *rps*L43 PCR- RFLP assay respectively.

7.1. Socio-demographic characteristics

Most participants in this study 157 (65.1%) lived in urban settings (Table 6.6.). There was a strong association between area of residence and HIV infection (p=0.001). The urban prevalence was 19.9% (48) whereas that of the rural was 5.8% (14) (Table 6.6).

There was no any difference in drug resistance across age groups between previously treated patients. Previous treatment was also significantly associated with HIV infection where a higher proportion of previously treated patients were observed to be HIV positive (p=0.023). This probably implies those new cases with HIV positive and this could have resulted in unsuccessful treatment outcome.

7.2. Drug resistance pattern

In this study, the prevalence of anti-TB drug resistance to at least one drug among newly diagnosed pulmonary TB patients in the Shashemene area of Southeast Ethiopia was found to be 19.8% while MDR was 0.9% identified in only two patients. Almost all drug resistance patterns, with the exception of Rifampicin and MDR, were significantly higher than in a previous report for a larger urban center, Addis Ababa (Demissie *et al*., 2001), but similar

with that of a more recent study for the same city (Dawit Asmamaw, MSc thesis, AAU/AHRI, 2005).

7.2.1. INH resistance

The prevalence of INH resistance was higher in this study compared to previous studies in Addis Ababa (Demissie *et al*., 2001). INH is given in both the intensive and the continuation phases of TB treatment. Particularly in the continuation phase, INH is given only with EMB. Since EMB is a relatively weak anti-TB drug (Zhang, 2005), it may not be effective to prevent development of resistance to INH. According to Woldemeskel *et al*., 2005, increased resistance to INH and RMP appeared to be higher in patients treated with 3 fixed dose combination (FDC) anti-TB drugs. However, this is not probably the reason for the increased INH resistance in this study since the increase in INH resistance was not accompanied by increase in RMP resistance (Table 6.2).

7.2.2. Rifampicin resistance

The level of Rifampicin resistance in our study, unlike with other types of resistance, was similar with previous findings (Bruchfeld *et al*., 2002). This could be attributed to the DOTS program implemented in the area. In the DOTS program, Rifampicin is given in the intensive phase under direct observation, together with at least three drugs, for category I patients. Moreover in the continuation phase, Rifampicin is spared. This fact, together with relatively lower rate of mutation developed against RMP and the rather relatively recent introduction of the drug in Ethiopia DOTS program could have resulted in a low level of resistance.

It was noted that different types of mutations to RMP result in different fitness levels. This situation creates differences in probabilities of transmission (Hailemariam *et al.*, 2004). Probably, the type of mutation frequently observed in the area could be one that renders strains ineffective in getting transmitted. However, further work is required on molecular characterization of RMP resistant isolates identified in the country.

A recent report in 2004 has shown that the prevalence of MDR-TB among newly diagnosed TB patients in Addis Ababa was 2.7% (Eyob *et al.*, 2004), which was higher compared with our study (0.9%). The difference in MDR prevalence between our study and that of Eyob *et al.*, 2004, could possibly be due to the methodologies used design of the studies, and the

study area as well. There is difference in the methodology between the two studies. Although both studies applied the proportion method, our study used Middlebrook 7H10 with 10% OADC enrichment while that of Eyob *et al.*, 2004, used Lowenstein Jensen medium (LJ) for determination of sensitivity pattern. When LJ is used for determination of anti-TB drug sensitivity test and the medium is heat sterilized together with the drugs added, the procedure might lead to inactivation of the drugs. Use of 7H10, on the other hand, is relatively better in maintaining the efficacy of the anti-TB drugs. In this study, drugs were added to the medium after heat sterilization of the medium and when temperature decreased to around 52^{0}C. Although the amount of drug added to LJ is increased to compensate for this phenomenon, it needs a tight control of temperature that would be difficult for some settings such as ours. This inactivation of anti-TB drugs in the medium is more common for RMP than for other drugs and may lead to overdiagnosis of RMP resistance (Heifets, 1991). Therefore, inactivation of the drugs could have resulted in high MDR prevalence (Asmamaw, 2005).

7.2.3. Ethambutol resistance

In this study, primary Ethambutol resistance was found to be 4.1% among new TB patients. However, this was not report on Ethambutol resistance during the surveys made before and up to 5 years after commencement of DOTS in Addis Ababa (1998). . In another study in Addis Ababa 9 years ago, a study which included all health centers and hospitals, the prevalence of EMB resistance was insignificant (Table 1.3.) (Demissie *et al.*, 2001). But, in 2001, the resistance rate was reported as 2.7% among newly diagnosed TB patients in Addis Ababa (Eyob *et al.*, 2004). Subsequently, there was a report of primary Ethambutol resistance among new TB cases of Somali region as 1.2% (Said, 2001). Thus, a significant increase in Ethambutol resistance has been observed in different parts of Ethiopia within short period of time. However, the recent report from Addis Ababa shows that the prevalence of EMB resistance to be similar with our study (Asmamaw, 2005).

One possible explanation for increased EMB resistance could be an increased defaulting rate from TB treatment in the continuation phase, the phase when EMB is administered. A considerable number of patients were also reported to default on treatment in the continuation phase. This could cause functional monotherapy and hence resistance. Another explanation for increased EMB resistance is that even if patients follow the recommended

continuation phase without defaulting, they would be receiving only INH and EMB. Previous reports and this study have shown that INH resistance is very common in the study area. Therefore, patients with primary INH drug resistance will undergo EMB monotherapy resulting in EMB resistance and this explanation is supported by the data in the previous studies (Table 1.3). Although the numbers are few, 50% of primary EMB resistant strains were also resistant to INH and STM. The same result was reported by Asmamaw (Asmamaw, 2005).

7.2.4. Streptomycin resistance

Streptomycin resistance was the common type of drug resistance observed in our study (16.6%). Any type of STM resistance has increased significantly compared to the surveillance in 1998. This drug has been in use since the beginning of TB chemotherapy. Thus a higher rate of STM resistance is developed to STM development is expected to, its long usage as an anti-TB drug (Hailemariam *et al.*, 2004).

7.2.5. Combined resistance (HS, HSE, SE, HRS)

In this study, the prevalence of drug resistance to more than one drug was 10.5%. This is much greater than in any of the previous reports from different parts of Ethiopia. Approximately 70% of this multiple resistance is combined resistance to INH and STM and 17% is due to combined resistance to INH, STM and EMB. This indicates that development of combined resistance to INH and STM is a frequent type of multiple resistance. This type of resistance could lead to further multiple resistance (such as HSE and HRSE) through amplification of the resistance.

To account for the high rate of multiple resistance observed in this study, it would be important to look back into the continuation phase where INH and EMB are given for 6 months. In an area where there is high level of INH resistance, treatment with INH and EMB is not probably effective and will lead to further acquisition of INH resistance and/or EMB resistance. This is because Ethambutol is a bacteriostatic drug with low efficacy that may not effectively prevent development of resistance to INH. According to previous reports, Addis Ababa is known to have high level of Streptomycin and Isoniazid mono-resistance (Lemma

et al., 1984; Demissie *et al.*, 2001; Eyob *et al.*, 2004). Therefore, additional resistance to INH or STM will lead to combined INH and STM.

The proportion of mono-resistance to any type of resistance for that particular drug is showing a decline (e.g. STM mono-resistance out of any type of STM resistance). This could be as a result of drug resistance amplification where mono-resistant isolates are acquiring additional resistance and are thus changed to multi-resistant (double, triple or quadruple resistance).

7.2.6. Comparison of drug resistance between 1998 and 2007

Our result was compared with that of a similar study in 1998 by Meaza Demisie (Demissie *et al.*, 2001) keeping in mind that the two studies were done in geographically separate localities and different epochs. This work was chosen for comparison since the design was comparable with that of ours but with due recognition that results may not be extrapolated except in generalities. Both setups implemented the same National TB control program in urban settings with high HIV prevalence and this shared certain similarities.

It is shown in Table 1.3. that anti-TB drug resistance is significantly higher compared to the situation in 1998. However there seems to be no rise in prevalence of MDR-TB and Rifampicin resistance.

As shown in the Table 1.3. all types of drug resistance patterns were higher in this study than in the 1998 report except for MDR and RMP resistance. However the increase in any type of RMP (and hence MDR) and any type of INH resistance were not statistically significant. The difference observed for STM was significant when the sample size was representing the population.

There is a difference between the two studies since the media used in our study was Middlebrook 7H10 agar with OADC enrichment while in the 1998 report LJ was used. Sensitivity determination, as it was discussed above, has a tendency to over diagnose drug resistance. Considering this fact will even make the difference between the two studies larger since had the 1998 study used enriched 7H10 medium the rate of resistance would probably have been lower.

It can be seen that 78.6% (22/28) of INH resistance in this study, occurred as multiple drug resistance while this figure was only 46.7% (7/15) in the previous study. A similar increase was seen in case of STM where a higher proportion occurred as multiple drug resistance compared to the previous study (Table 6.2 and Table 1.3). This would suggest that amplification of resistance was frequent on INH mono-resistant and to some extent on STM mono-resistant strains with the strains acquiring additional resistance. Probably amplification of resistance was more common than acquisition of mono-resistance for INH.

A similar finding of progressive increase in any type drug resistance, with no significant rise in MDR, was reported from an African country, Ethiopia and Botswana. This increase over a period of around 10 years was statistically significant. The country is recognized for its strong DOTS program. Another study in Botswana has shown that drug resistance was not associated with HIV infection (Kenyon *et al.,*1999).

In the third global TB report, it was noted that in previously treated patients of DOTS implementing areas, MDR could be developed in sequential manner. In this report, it was observed that initial resistance to INH or STM is a gateway to multidrug-resistance. INH or STM resistance will be amplified to double STM and INH resistance and then triple INH, STM and RMP resistance. Finally Quadruple (and also MDR) drug resistance will be developed when resistance to RMP is developed (WHO/IUATLD, 2004).

In this study, we have seen the high anti-TB drug resistance patterns. Particularly the high multiple drug resistance was worth noting. The TB strains circulating in the environment have already begun a journey towards MDR-TB. It appears that drug resistant TB has moved forward one step. i.e. some drug sensitive strains have turned in to mono-resistant and majority of mono-resistant strains have changed to double STM and INH resistant.

The adequacy of DOTS, in circumstances where there is a significant drug resistance problem, was a concern by different investigators (Connix *et al.,* 1999; Kimerling *et al.,* 1999). Farmer *et al.,* 1998 were also arguing that in settings where there is a high mono-resistance rate, a mere DOTS program could create a selection pressure that facilitates the emergence of MDR-TB (Farmer *et al*., 1998).

7.3. Molecular Analysis of drug resistance genes

This study showed that a large proportion of INH and STM resistant *M. tuberculosis* isolates in Southeast Ethiopia. The amino acid 315 in the *katG* gene and amino acid 43 in the *rpsL* gene are the most responsible genes for development of drug resistance in INH and STM respectively. Based on the *katG315* MAS-PCR and *rpsL43* PCR-RFLP assay (Mokrousov *et al.,* 2002), it was confirmed that a simple and rapid tool for detecting with high probability the INH and STM resistance in *M. tuberculosis* clinical strains. 83.8% and 55.6% of *in-vitro* drug resistance was confirmed by polymerase chain reaction based mutation analysis for INH and STM drug resistant bacteria respectively. In 2002 a similar result was reported from Russia (Mokrousov *et al.,* 2002). In our study, mutation analysis done from culture and it is also applicable for direct detection from sputum samples, which should facilitate the adequate and timely choice of anti-TB therapy. However, it has been suggested that the *kat*G315 and *rps*L43 alteration alone may not always be clinically significant (Mokrousov *et al.,* 2002).

7.4. Determinants of drug resistance

In this study, no association was observed between HIV infection and drug resistance in newly diagnosed TB. This could suggest that drug resistant TB strains may cause TB in both immunocompromised and immunocompetent TB patients at a similar rate. However, the study has not compared the level of immunosuppression in the co-infected patients to confirm that the similarity in susceptibility to drug resistant TB strains in both groups is true for all levels of immunosuppression. Immunity is not much compromised in early stages of HIV infection. Thus, the study may be interpreted to indirectly argue against the hypothesis that drug resistant TB is less virulent and causes disease mainly in immunocompromised TB patients (Anton, 2001). Another possibility is, however, that there might actually have been an association between drug resistant TB and HIV, but that we failed to pick up this association since HIV patients with drug resistant TB patients die earlier than HIV negative TB patients with drug resistant TB. Not only could this phenomenon possibly account for lack of association between HIV and drug resistant TB, but it could also explain the higher proportion of drug resistance in HIV negative than in co-infected patients. The degree of

immunodeficiency among co-infected patients could probably also be not advanced since patients were selected based on smear positive microscopy. Co-infected patients with severe immunodeficiency tend to be smear negative on sputum examination. A limitation of the study, in this regard, is that the degree of immunodeficiency of the HIV-TB co-infected patients has not been determined. Also, the sensitivity profile of strains causing TB in smear negative HIV positive patients was not investigated.

In this regard, contradictory findings have been reported by different studies in Ethiopia. Our findings are similar to several others with those of many other research groups (Bruchfeld *et al.*, 2002; Eyob *et al.*, 2004). But, a 2001 report suggested an association between drug resistant TB and HIV (Anton, 2001). This finding could be a result of an outbreak of drug resistant TB in institutions such as hospitals (Anton, 2001). Later on, the patients transmitted it to HIV negative patients and the difference was masked (Palmero *et al*., 2003).

HIV patients with drug resistant TB die sooner after developing the disease, further diminishing the difference between the two groups (Fischl *et al.*,1992). Because of this the statistical difference that was seen between the two groups in 1998 could be non-existent during this surveillance.

History of previous treatment with anti-TB drugs was significantly associated with any type drug resistance (OR, 0.292). Previous treatment was also associated with multiple drug resistance (OR, 3.05). The association still existed when it was adjusted for HIV, age, occupation and BMI. This finding is in accordance with previous reports (Pritchard *et al.*, 2003; Eyob *et al.*, 2004;) and shows the selective nature of ineffective treatment. i.e. in patients who did not respond to the anti-TB drugs for one reason or other, the TB strains will be exposed to the anti-TB drugs. This increases the probability of selection where a drug resistant TB strain will dominate over the sensitive strains.

Previously treated TB patients could have drug resistant TB from the outset. Those patients with drug resistant TB have a tendency to have an unsuccessful treatment outcome, probably because of the failure to clear bacteria. This unsuccessful treatment in turn would probably lead to the acquisition of resistance to more of the individual drugs (amplify already existing drug resistance status) and lead them to multiple drug resistance. This is probably reflected in

this study since previously treated patients were > 4 times more likely to have multiple drug resistance compared to the new TB patients (p=0.006).

7.5. Misclassification of patients and its implication on drug resistance

Misclassification of previously treated patients as new cases is a possibility in such a study. This situation could result in overestimation of drug resistance prevalence among the study participants. However, there are two pieces of evidence that this is most probably not the case. First, classification of patients as new or previously treated cases was very stringent. We used standard WHO questionnaires that were translated into Amharic and pre-tested. There was quality control for information collected through questionnaires where every twentieth patients was re-interviewed by another health professional. There were 5 misclassified patients identified and these errors were also corrected. After the sensitivity pattern had been determined, those individuals with drug resistant TB were contacted and their previous treatment status was re-confirmed. We had only 24 previously treated patients in the analysis, suggestion that the patients were classified correctly. Secondly, previously treated patients were more likely to be HIV positive and to have drug resistant TB. Therefore, misclassifying previously treated individuals as new cases increases the proportion of HIV patients with drug resistant TB. Had this been the case in this study, we should have seen higher proportion of drug resistant TB in HIV patients. We have observed is the opposite.

7.6. Interaction between HIV and TB: Implication on TB Control

The prevalence of HIV among all smear positive TB patients was 25.7%. This figure was 22.6 % in new cases and 54.2% in previously treated patients. This difference was statistically significant (p=0.02). Previously treated TB patients might have HIV when diagnosed and have had poor treatment outcome. This could result in drug resistance. It could be implied then HIV is probably an indirect factor for acquired drug resistance. This also needs further verification where previously treated TB patients should be studied and the interaction between drug resistance pattern and HIV determined.

The prevalence of HIV in this study (25.7 % for all cases and 22.6% among newly diagnosed TB patients) is less compared to a previous report among smear positive TB patients in Addis Ababa, 45.3% (Demissie *et al.*, 2001). This could show a real decline in the prevalence of

HIV in the smear positive TB population. The WHO estimation of TB/HIV co-infection rate has also declined from 29% in its 2004 report to 21% in 2005. Another possibility is that TB/HIV co-infected patients during the conduct of this study might have been more immuno-compromised and presented with smear negative TB. According to the latter explanation, even if the proportion of HIV co-infected TB patients is somewhat similar with the previous ones HIV co-infected patients might have not been included in the study because of their clinical presentation. Still another possibility is that HIV co-infected TB patients might have guessed their HIV status from high risk behavior and refused to consent for the study. However, this is unlikely since the country is in the verge of distributing anti-retroviral drugs for free and some NGO's have already started distributing the drugs for eligible patients. Additionally, there is a lot of work being done to create awareness about the disease and avoid stigma associated with the disease.

It was difficult to calculate proportion of refusals since in the registry books, there were patients that were not eligible to our study. Therefore, the denominator was not reliably identified. However, according to information from nurses that recruited the patients, around 95% of patients have consented.

7.7. TB strains in smear positive pulmonary TB patients of Shashemene area, Southeast Ethiopia

It was observed that 8.3% (20/241) of the isolates were sensitive to TCH. This sensitivity suggests that the strain could be a species other than *M. tuberculosis (such as M. bovis)*. Sensitivity to TCH was the most reliable method so far reported in species identification (Collins and Levett, 1989; Parsons *et al.*, 2002). All species of tuberculosis in the *M. tuberculosis* complex with the exception of classical *M. tuberculosis* are reported to be sensitive to TCH.

All the strains sensitive to TCH and 20 randomly selected INH and TCH resistant strains were subjected to PCR amplification. All the strains were amplified by RD4 primer of *M. tuberculosis* suggesting that *M. bovis* was not present. Probably the 20 TCH sensitive strains are more likely to be Asian type *M. tuberculosis* or *M. africanum* (Yates *et al.*, 1984).

8. STRENGTHS OF THE STUDY

1 This study was initiated to answer the question, whether MDR-TB contributed to the reported high mortality rate in, hospitalized TB patients or not.

2 The study is the first to address the prevalence of anti-TB drug resistance and HIV/TB co-infection in a major urban centre at the cross roads of probably the busiest highway in the country, Shashemene, and in its adjoining rural towns.

3 Identification of previously treated patients was done with a structured questionnaire. Additionally the utmost effort was exerted in checking the archives of the treatment centre to check registration of the research participants for TB treatment. Moreover, the quality control of the data was checked by re-filling questionnaires for every tenth patient.

4 Samples were collected from four health centres and a Hospital. This, we believe, provided sufficient representativeness of the sample for the Shashemene area.

5 Molecular method of drug resistance confirmation were included for INH and STM to confirm conventional culture techniques drug resistance study.

6 Quality control of the drug resistance assays was checked in the laboratory using appropriate reference strains (ATCC35835, ATCC35836 and ATCC35838)

9. LIMATTION OF THE STUDY

1. The study does not provide information on smear negative TB patients.

10. CONCLUSIONS

1 Anti-TB drug resistance, especially resistance to multiple drugs, any type of Ethambutol resistance and any type of streptomycin resistance in the Shashemene, Southeast Ethiopia is relatively high when compare to other studies in the country.

2 Prevalence of rifampicin and multi drug resistance among new cases was relatively similar with the previous reports in the other parts of the country in 2001.

3 HIV was not confirmed to be associated with drug resistance among new cases of pulmonary TB in the area. However, HIV could result in drug resistance indirectly through ineffective treatment.

4 *Mycobacterium bovis* is probably not a common cause of smear positive pulmonary tuberculosis in the Shashemene area, Southeast Ethiopia.

5 83.8% and 55.6% of *in-vitro* drug resistance was confirmed by polymerase chain reaction based mutation analysis for INH and STM drug resistant bacteria respectively.

11. RECOMMENDATIONS

1 Six months of treatment with EMB and INH in smear positive TB patients is probably not effective in areas where there is higher level of INH resistance. Therefore, operational research investigating other alternative regimens (such as RH, RHE) in the continuation phase will be important

2 Characterization of the molecular pattern of Rifampicin resistant isolates in Shashemene area, Southeast Ethiopia is probably necessary. This would help to assess the fitness of Rifampicin resistant strains to get transmitted and hence the use of the drug in the continuation phase.

3 There is a need also to determine the extent of drug resistant TB in smear negative and extra-pulmonary cases of TB.

4 Smear positive TB patients with delayed smear conversion and re-treatment groups are better managed putting into consideration their sensitivity pattern since significant proportion of patients have drug resistance.

5 Second-line anti-TB drugs are required at this point where the resistance pattern in the area is increasing and progressively leading to MDR-TB. This would be appropriate putting into consideration the prevalence of multiple drug resistance among new cases which is greater than 10%.

6 It would be appropriate to follow patients from whom *Mycobacterial* isolates were studied and correlate their treatment response pattern with their drug resistance pattern.

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ANNEX I. Distribution of any type drug resistance among newly diagnosed TB patients in Southeast Ethiopia survey covered area (n=217).

No.	**Name of the centre**	***Sensitive N (%)***	***Resistant N (%)***	***Total N (%)***
1	Aje	1 (0.5)	1 (0.5)	2 (0.9)
2	Alaba	1 (0.5)	0	1 (0.5)
3	Arsi-Negelle	14 (6.5)	3 (1.4)	17 (7.8)
4	Asasa	1 (0.5)	0	1 (0.5)
5	Awassa	21 (9.7)	8 (3.7)	29 (13.4)
6	Bale	1 (0.5)	0	1 (0.5)
7	Delecha	1 (0.5)	0	1 (0.5)
8	Dodola	1 (0.5)	0	1 (0.5)
9	Kofelie	4 (1.8)	1 (0.5)	5 (2.3)
10	Kuyera	1 (0.5)	1 (0.5)	2 (0.9)
11	Melkaoda	1 (0.5)	0	1 (0.5)
12	Siraro	2 (0.9)	0	2 (0.9)
13	Shashemene	114 (52.5)	27 (12.4)	141 (65.0)
14	Shegulie	1 (0.5)	0	1 (0.5)
15	Suaska	1 (0.5)	0	1 (0.5)
16	Wondo Genet	7 (3.2)	1 (0.5)	8 (3.7)
17	Zeway	2 (0.9)	1 (0.5)	3 (1.4)
	Total	***174 (80.2)***	***43 (19.8)***	***217 (100)***

p-value= 0.951

ANNEX II.

Information Sheet

Armauer Hansen Research Institute (AHRI)
School of Graduate Studies (AAU/Science Faculty)

Name of the Principal Investigator**: **Desta Arega Anore

Name of the organization**:** **Addis Ababa University-Science Faculty**

Name of the sponsor**:** **School of Graduate studies/AAU and**
Armauer Hansen Research Institute (AHRI)

Information Sheet prepared for participants from Shashemene town in research project that study the Molecular Epidemiology and Drug Resistance pattern of *Mycobacterial* Isolates Among HIV positive and HIV negative TB patients.

This information sheet is prepared by groups of research investigators whose main aim is to study anti-TB drug resistance patterns and the type of *M. tuberculosis* strains circulating in and around Shashemene area. The investigators include final year MSc graduate student from Science Faculty of Addis Ababa University, a senior researcher from AHRI and an advisor from Science Faculty/AAU and physician from Kuyera Hospital.

Purpose:

The purpose of this research is to study the anti-TB drug resistance patterns of *Mycobacterial* isolates among HIV positive and HIV negative TB patients and the type of *M. tuberculosis* strains circulating in and around Shashemene area. It is well known that TB is one of the common diseases in our population. If we have some understanding of how these microorganisms develop anti-TB drug resistance and the type of *M. tuberculosis* strains circulating in the area, it has great significance in development of effective control activities in and around Shashemene area.

According to various studies, drug resistance *M. tuberculosis* strains have emerged, posing a major challenge to TB control efforts. The incidence of drug resistant TB has increased in many parts of world, not only in developing countries but also in industrialized countries. So if we understand whether HIV increases drug resistance or not, will help us how to use this knowledge in controlling TB and HIV.

Procedure:

To assess anti-TB drug resistance patterns and *M. tuberculosis* strain types among HIV positive and HIV negative TB patients, we invite you to take part in our project. If you are willing to participate in this project, you need to understand and sign the agreement form. Then, you get general clinical examination by physician of the Hospital/Health Center to check your health conditions. Based on this examination, the physician has a responsibility to recommend whether you are capable or not in participating in the project. For example age less than 18 years, prolonged liver or kidney disease or in case of sever anemia the physician may not recommend you for participation this research. However, if you are unable to participate in the study by the recommendation of the physician or based on your interest, it has no effect in any way in obtaining the regular health services delivered at the Hospital/Health center.

For laboratory examination, sputum sample and 10 ml of blood will be taken from vein in your hand for HIV screening. The cost for clinical examination will be covered by the project. We are informed and also agreed to get the voluntary counseling and testing service for HIV, since it give us a benefit for planning how to manage our future health status. We will get the counseling and testing service free of charge. All the clinical and laboratory examination results will be kept confidential using coding system where by no one will have access to your clinical and laboratory results.

Risk and Discomfort

By participating in this research project, you may feel that it has some discomfort but this will not be too much as you is coming to clinic for routine TB care. There is no major risk in participating in this research, but the minor bleeding that may occur during blood collection will be avoided, as the procedure is carried out by experienced health professionals in the health center based on the standard good clinical practice.

Benefits

If you participate in this research, you may not get direct benefit but any treatable disease conditions diagnosed during the study period will be taken care of at no charge to you. In addition, your participation is likely to help us in understanding the mechanism of anti-TB

drug resistance patterns of Mycobacterial isolates and *M. tuberculosis* strain types in HIV positive and negative TB patients in our population and may benefit in the future to design control strategy of the disease in our society.

Incentives

You will not be provided any incentives to take part in this research. However, you will be reimbursed 15 Birr for your lost time and your travel expense during participation in the project.

Confidentiality:

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept under lock and key, and it will not be revealed to anyone except the principal investigator and your clinician.

Right to refuse or withdraw

You have full right to refuse from participating in this research if you do not wish to participate; and this will not affect your treatment or health services you get at this health center in any way. You have also full right to withdraw from this research at any time you wish to, without losing any of your rights as a patient in the health center.

Whom to contact

This research project was reviewed and approved by AHRI/ALERT Ethical Committee and by National Ethical Research Committee (NERC). This committee is the highest body in the country to approve such research plans. The main task of both institutional and national ethical committees is to make sure that research participants are protected from harm. If you want more information and check about this project, you can contact the Chairman of the AHRI/ALERT Ethical Committee **Dr. Howard Engers**, **AHRI, Tel. no. 710288, Addis Ababa** and NERC **Dr. Yimtubzinash W/ amanuel, Tikur Anbessa Hospital/ Medical Faculty, Tel. no. 511211 Addis Ababa.**

If you have any questions contact any of the following individuals and you may ask at any time you want:

1. **Desta Arega, AHRI, Tel. no. 0911840604 Mobile.**
2. **Dr. Abraham Aseffa, AHRI, Tel. no. 0113211334 Addis Ababa.**
3. **Dr. Mekuria Lakew AAU, Tel. No 0911217999 Mobile.**

ANNEX III: CONSENT FORM

Name of study participant ---------- Age---------- Sex----------

Physician Name---------- Site /Health center ----------PIN ----------

I have been informed about a study that plans to investigate the **Anti-TB drug resistance patterns and *M. tuberculosis* strain types among HIV-positive and negative TB patients,** which will help in understanding the *M. tuberculosis* strain types in HIV-positive and negative TB patients and their drug resistance patterns to anti-TB drug and assists in developing better TB and HIV control method in the country. For this study I have been requested to give a sputum sample as part of the routine care for TB and 10 ml of blood for HIV screening. I have been informed that I will get pre-test HIV counseling before giving the blood. The investigator has briefed me that there are no major risks associated with the sampling procedure except very minimum bleeding and to avoid the possible risks, blood collection will be done by experienced health professionals according to the established aseptic procedure in clinical care. I have been informed that there is no direct benefit provided to me, but an amount of 15 Birr will be paid to each eligible participant towards reimbursement of their transport expense and a nominal compensation for their time to participate in the study. If I will decide to know the result, I will get post-test counseling. If I am HIV positive, the investigators of this project will facilitate referral to the voluntary counseling and testing (VCT) centers to obtain best possible standard care provided in these centers. The investigator also informed me that all the laboratory results would be kept confidential. Moreover, I have also been well informed of my right to withdraw from participating in this project and that my actions will have no impact on the overall management of my conditions. I have been given enough time to think over before I signed this informed consent. It is therefore, with full understanding of the situation that I gave my informed consent and cooperate at my will in the course of the conduct of the study.

Name (participant) ----------Signature ----------Date ----------

Name (investigator) ----------Signature ----------Date ---------

Name (Witness) ----------Signature ----------Date ----------

Form 1-CLINICAL INFORMATION

Date of interview_____(EC) TB center No_____ Lab No _____

A. IDENTIFICATION OF THE PATIENT

Name_____

Age:_____ Sex Weight Height

Marital status; Not married Married Divorced

Address: Wereda_____ Kebele_____House No_____Phone No_____

Educational status _____ Occupation_____

Date of Sputum Collection: _____ Date of blood Collection: _____

B. CURRENT CLINICAL PRESENTATION

Manifestation (put x)	YES	NO	Duration (if Yes) (in weeks)
1. Hemoptysis			
2. Fever			
3. Chest pain			
4. Cavity (If chest X-ray taken)			chest X-ray not taken
5. Others (specify)			

C. PREDISPOSITION FOR TB

1. Diabetes; Yes No

If yes, is it being treated ? Yes No

2. Cigarette smoking; Yes No

if yes, how many cigarettes per week do you smoke?_____

if yes, when was the last time you smoked ? _____

How long have you smoked ? _____

3. Do you drink alcohol ? Yes No

if yes, when was the last time you drunk alcohol ? _____

If your answer to question No. 3 is yes, fill the following table

Type of the alcohol (put "x")	Tella	Tej	Araki	Beer/Drought	Other (specify) _____
amount of alcohol in average (per week)					

How long have you taken alcohol ? _____

Hospitals and prisons are places where drug resistant TB is acquired.

4. Do you have Previous imprisonment for any disease? Yes No

If your answer is yes,

a.When was it? month/year _____

b. Which ward were you admitted ? _____

c. How long were you admitted ? _____

d. Were there chronically coughing individual ? Yes No

5. Do you have Previous imprisonment for any disease? Yes No

If your answer to is yes**,**

a.When was it? month/year _____

b. How long were you imprisoned? _____

c. Were there chronically coughing individual ? Yes No

D. HISTORY ON PREVIOUS TB

***D1*. Previously treated for TB**? *Yes* *No*

If the answer is **no**, go to **D2** ; if **yes**, go to **D3**.

D2. Standardized history

1 How long have you been sick?_____Weeks

2 Have you had the same symptoms prior to this episode? Yes ☐ No ☐

3 Have you had other symptoms of lung disease prior to this episode (Hemoptysis, chest pain, cough)? Yes ☐ No ☐

4 Have you had X-ray examinations prior to this episode? Yes ☐ No ☐

5 Have you had sputum examinations prior to this episode? Yes ☐ No ☐

6 Have you had drug treatment for more than one month? Yes ☐ No ☐

if yes, what were the names of the drugs_____

☐ ☐

7 Have you ever received injections for more than four weeks? Yes No

Did the patient remember previous treatment for TB after these questions**?** Yes ☐ No ☐

If yes continue with **D3**

D3. Information about previous treatment

1. Where was the patient treated?_____
2. When was the patient treated?_____
3. How long was the patient treated?_____weeks
4. Which drugs were used for treatment?_____
5. How many courses of treatment were given?_____
6. Outcome of the last treatment according to the patient.

Cured ---------- Not cured ---------- Unknown ----------

E. FINAL DECISION

E1. Patient has been previously treated for TB for more than one month

Yes ☐ (answer to question D1 or D2)

No ☐ (answer to D1 and D2)

Doubtful ☐

E2. If yes, what was the outcome of previous treatment?

Cured/treatment completed ☐ Failed ☐

Defaulted ☐ Chronic ☐

Unknown ☐

Signature of Responsible Proffesional Health worker(interviewer)_____

F. FOR PREVIOUSLY TREATED PATIENTS

F1. Remission period in months (free from symptoms after the last treatment) weeks ☐

F2. Treatment previously taken

1. treatment taken previously (put "x")			
Drugs	**Taken**	**Not taken**	
Isoniazid			
Streptomycin			
Rifampicin			
Ethambutol			

2. Treatment regimen previously used

2.1. Intensive phase (specify) A. Loose ☐ B. 2FDC ☐ C. 3FDC ☐

2.2. Continuation phase (specify) A. Loose ☐ B. 2FDC ☐

G. HIV STATUS (IF KNOWN PREVIOUSLY) A. Positive ☐ B. Negative ☐

If positive; is he/she getting anti retroviral drugs: yes ☐ No ☐

FORM 2- MEDICAL RECORDS

A.1 After extensive checking through the medical files and other documents available in the health center within the past two years, have you discovered that the patient has been registered for tuberculosis treatment before?

No ☐ Yes ☐

If yes, what was the outcome of the last course of chemotherapy?

Cured ☐ Treatment completed ☐

Defaulted ☐ Failed ☐

Transferred-out ☐

A.2. Remission period in months (free from symptoms after the last treatment) weeks ☐

A.3. Treatment previously taken

1. treatment taken previously (put "x")			
Drugs	**Taken**	**Not taken**	
Isoniazid			
Streptomycin			
Rifampicin			
Ethambutol			

2.Treatment regimen previously used

2.1. Intensive phase (specify) A. Loose ☐ B. 2FDC ☐ C. 3FDC ☐

2.2. Continuation phase (specify) A. Loose ☐ B. 2FDC ☐