

**DRUG RESISTANCE PATTERNS OF TUBERCULOSIS AMONG
RE-TREATMENT CASES IN St PETER'S TB SPECIALIZED
HOSPITAL, ADDIS ABABA**

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DECLARATION

I the undersigned, declare that this thesis is my original work, has not been presented for a degree in other university and that all source materials used for the thesis have been duly acknowledged.

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List of Abbreviations

AHRI	Armauer Hansen Research Institute
ARTI	Annual Risk of Tuberculosis Infection
ATCC	American Type Culture Collection
BCG	Bacille Calmette- Guerin
Bp	Base pair
C	Cytosine
DMF	Dimethyl formamide
DNA	Deoxyribose nucleic acid
DOT	Directly Observed Therapy
DST	Drug Sensitivity Test
ECL Kit	Enhanced Chemiluminiscence Kit
EDTA	Ethylenediaminetetra- acetic acid
EH	(Ethambutol/ Isoniazid)
E	Ethambutol
EPTB	Extra Pulmonary Tuberculosis
FDC	Fixed Dose Combination
G	Guanine
HIV	Human Immuno deficiency Virus
H	Isonicotinic acid hydrazide (Isoniazid)
IS	Insertion Sequence
IUATLD	International Union Against Tuberculosis And Lung Disease
LJ	Lowenstein Jensen
McF	MacFarland
MDR	Multidrug Resistance
MOH	Ministry Of Health
MTT	3-(4,5- Dimethylthiazol –2-Yl)-2, 5- Diphenyl Tetrazolium Bromide
OADC	Oleic Acid Albumin Dextrose Catalase
OD	Optical Density

PANTA	Antibiotic mixture (Polymyxin B, Amphotericin B, Nalidixic acid, Trimithoprim, Azlocillin)
PBS	Phosphate Buffered saline
PMNS	Polymorphonuclear Cells
PTB	Pulmonary Tuberculosis
Z	Pyrazinamide
R/F/D	Relapse/Failure/ Defaulter
RFLP	Restriction Fragment Length Polymorphisms
RH	Rifampicin/ Isoniazid
RHZ	Rifampicin /Isoniazid/ Pyrazinamide
R	Rifampicin
RNA	Ribose Nucleic Acid
RODU	Relative Optical Density Unit
RPM	Round per minute
SCC	Short Course Chemotherapy
SDS	Sodium Dodecyl Sulphate
S	Streptomycin
TB	Tuberculosis
TBE	Tris- Borate EDTA
TCH	Thiophene 2 Carboxylic acid Hydrazide
TE	Tris- EDTA
TLCP	Tuberculosis and Leprosy Control Programme
TNF	Tumor Necrosis Factor
WHO	World Health Organisation

ABSTRACT

Tuberculosis (TB) is a major public health problem worldwide. About 3.8 million TB cases were reported to WHO in the year 2001 from the 8.5 million estimated new cases. In Ethiopia the estimated incidence of all cases of tuberculosis has reached 292 per 100000 populations. This figure placed the country to rank 10th among the 22 high burden countries.

The resurgence of tuberculosis has been accompanied by high frequency of drug resistant strains from all over the world. In most TB patients drug resistance predominantly arises as a result of multiple interruptions of treatment. To avoid this problem fixed-dose combinations (FDCs) tablets are now recommended by WHO and IUATLD. However, in FDC formulations the bioavailability of the component drugs, and especially of rifampicin, may be reduced. Simple, rapid and inexpensive methods of detecting drug resistant tuberculosis are also essential for effective treatment.

This study has the objectives of assessing the prevalence of drug resistance among re-treatment cases after introduction of Three Fixed Dose Combination (3FDC) therapy for tuberculosis; evaluation of MTT assay for direct detection of rifampicin resistance and analysis of the RFLP pattern of the different TB strains. Single sputum samples were collected from 100 smear positive re-treatment TB cases who attended St Peter's TB specialized Hospital between 21 December 2001 and 15 October 2002. The sputum samples were cultured on Lowenstein- Jensen (LJ) media and 7H9 broth. Drug sensitivity was done on 7H10 agar using the proportion method. Broth media (7H9) supplemented with PANTA and Oleic acid Dextrose Catalase (OADC) were used for MTT assay. Formazan production was quantified by measuring the optical density (OD) at 570nm. Relative optical density unit (RODU) was calculated and resistance was defined as RODU > 0.5 and susceptibility as RODU < 0.2.

Among the 89 culture positive isolates, 75 were tested for drug sensitivity pattern. Totally 58.7% of the isolates were resistant to one or more drugs. Isolates resistant and partially resistant to isoniazid were found to be 42.7% and 6.7% respectively. Resistance to rifampicin was 33.3%. Isolates resistant and partially resistant to streptomycin were found to be 21.3% and 12% respectively. The percentage of isolates resistant to ethambutol was 9.3% while 25.3% were partially resistant. Multidrug resistant isolates (MDR) were observed in 29.3% of the patients. Patients who had a history of treatment with 3FDC had a statistically significant higher rate of resistance to isoniazid ($P < 0.05$) and rifampicin ($P < 0.05$) compared to those treated with loose drugs. Direct MTT assay identified 30.7% isolates as rifampicin resistant and 69.3% as susceptible within three weeks of time. Comparing MTT assay to the proportion method resulted in 97.3% matching. RFLP analysis of ten isolates showed the presence of eight unique patterns. Two isolates showed the dominant type of RFLP profile existing in Ethiopia. Contrary to our expectation, patients treated with 3FDC regimen had more rifampicin and isoniazid resistant isolates than those treated with loose drugs. This is a good indicator for a more systematic study to evaluate the efficacy of the 3FDC drug formulation. There has been a marked increase in drug resistant tuberculosis. Therefore, nationwide drug resistance surveillance with a larger number of samples is needed to monitor drug resistance tuberculosis in the country.

1 Literature Review

1.1 The Bacteria

M. tuberculosis is a pathogenic bacteria that belongs to the class actinomycetes, order actinomycetales and family mycobacteriaceae. The genus mycobacterium includes obligate parasites, saprophytes and intermediate forms.

It is typically slender, straight or slightly curved and rod in shape. It occurs as a single cell or in a thread like form. The size of the bacillus ranges from 0.3µm to 0.6µm in width and from 0.5µm to 4.0µm in length. It is slow growing, non- encapsulated, non- spore forming, non- motile and lipid rich organism (Salyers and Whitt, 1994).

The cell wall of *M. tuberculosis* is similar to that of gram-positive organisms except that it has higher lipid content. The lipids are long chain fatty acids and present difficulties in staining. Therefore, heat or increased concentration of stain is required to achieve staining (Laidlaw, 1989). This unique cell wall of mycobacteria is also responsible for its resistance to the lethal effects of acids, alkalis and detergents. This is a characteristic fully exploited for isolation of the organism from other bacteria for culturing (Evans, 1998). *M. tuberculosis* optimally grows at a temperature range between 35⁰C-37⁰C (Grange, 1990).

Based on the now available whole genome sequence of *M. tuberculosis*, it is suggested that the common ancestor of tubercle bacilli resembled *M. tuberculosis* and could have been a human pathogen (Brosch *et al.*, 2002). This finding contradicts the hypothesis that the human *M. tuberculosis* evolved from *M. bovis* during domestication of cattle.

1.2 Transmission of tuberculosis

Tuberculosis is spread from person to person by aerogenic transmission. The source of infection is a patient with pulmonary TB who coughs and spreads tiny droplets. A single cough may produce up to 3000 droplets where each one contains one or more tubercle bacilli (WHO, 2000b). A person is considered infected with *M. tuberculosis* if she or he converts from negative to positive on the tuberculosis skin test (Sudre *et al.*, 1992). Twenty five to fifty percent of the household contacts of patients with active pulmonary disease become infected (Grzybowski *et al.*, 1975).

Under normal circumstances only a small proportion (about 10%) of all individuals who are infected by the tubercle bacilli develop the disease in their lifetime. Vast majority (90%) of people exposed to the bacteria except those with HIV infection do not develop the disease (WHO, 1996).

1.3 Pathology

Healthy adult exposed to relatively low numbers of bacteria generally clears them before appreciable damage to the lung occurs. But if the phagocytic cells do not clear the infection, new T cells, polymorphonuclear cells (PMNs) and more macrophages continue to be attracted to the area where bacteria are growing (Salyers and Whitt, 1994).

In some cases where the phagocytes fail to kill the bacteria, the T cells and macrophages wall off the growing lesion with a thick fibrin coat. The walled off lesion is called tubercle. Tubercles eventually calcify, giving rise to the hard-edged lesions visible in chest x-rays. Phagocytes unsuccessfully trying to kill the bacteria cause considerable damage to lung

tissue by releasing lysosomal enzymes and by producing tumor necrosis factor (TNF). TNF causes tissue damage and is probably responsible for the weight loss that occurs in people with tuberculosis. Initially the areas where bacteria are growing have a thick, cheese like appearance (caseous necrosis). As bacteria continue to grow and phagocytes continue to enter the area, the necrotic region becomes much more liquid. A person with liquefied lesions is more contagious than lesions in caseous necrosis (Sudre *et al.*, 1992).

1.4 Clinical Manifestation

Pulmonary tuberculosis patients usually have weight loss and productive cough for more than three weeks. Symptoms like haemoptysis, chest pain, dyspnea, fever, night sweat, and anorexia have also been shown to be common among TB patients (WHO, 1996). Chest X-ray findings such as infiltrates and cavitation are also confirmatory indicators of tuberculosis infection (WHO, 1996).

1.5 Global Epidemiology of tuberculosis

Tuberculosis is the most frequent cause of death from a single infectious disease in persons aged 15-49 years. About 8.4 million people develop active tuberculosis every year and 2.3 million die of it. It is estimated that 200 million additional people are at risk of developing the disease in the next 20 years, if the current trends are conserved (Miller and Schieffelin, 1998)

Report from 183 countries shows that there are 3.8 million cases (62 per 100000 population) around the world. Nearly 42% of these cases are sputum smear positive. Compared to WHO estimation of new cases (8.5 million) the case notification from the

above countries is surely very low. The global incidence of TB is growing at 0.4% each year. More rapid growth was observed in sub-Saharan Africa due to the spread of HIV and in countries of the former Soviet Union. The estimated TB incidence is high in African countries. Treatment success under DOTS for the 2000 cohort was 82% on average and it is below the average (72%) for African region (WHO, 2003).

The DOTS strategy has been the principal response to the global TB epidemic for the past decade. By the end of 2001, DOTS had been adopted by 155 countries and was available to 61% of the world Habitants. DOTS programmes between the start of 1995 and the end of 2001 diagnosed more than ten million patients. Of these over five million were smear-positive (WHO, 2003).

1.6 Tuberculosis in Ethiopia

The burden of TB in Ethiopia is one of the highest in the world and based on estimated number of cases the country is placed to rank 10th among the 22 high burden countries (HBC) (WHO, 2003).

In the year 2001, the Tuberculosis and Leprosy Control Programme (TLCP) registered 94,957 TB cases from the DOTs implementing areas and reported 33,028 of them to be new smear positive PTB cases. This represents a case notification rate of 173 and 60 per 100,000 population for all forms of TB and smear positive cases respectively (MOH, 2002).

In 1996, the DOTs programme covered 15 zones with a population of 15 million. By the end of the year 2001, the programme covered 56 zones in nine regions and two

administrative councils. These zones represent 76% of the country where 55 million (85%) of the people live (MOH, 2002). A report presented at the tenth annual review meeting TLCP Ethiopia (18 to 20 September 2002) (Table 1) show the increase in the incidence of tuberculosis (TLCP, 2002).

Table 1 Tuberculosis case notifications in Ethiopia. Period: 2001- 2002 (Source, TLCP, 2002)

Place	Total notified cases	All new cases	Pulmonary smear positive cases starting treatment				PTB-ve	EPTB
			New smear +ve	Relapse	Failure	Default		
A.A	12712	12245	3911	390	30	47	4591	3743
Ethiopia (Total)	107626	105250	35915	1554	303	519	32197	37138

Table 2 shows WHO estimation of tuberculosis burden in Ethiopia and the incidence of all forms of TB are at 260 per 100,000. The WHO estimation is higher than case notification rate estimated by MOH since it considers factors like underreporting and under diagnosis by health institutions, the prevalence in non-DOTs areas, and the problem of passive case detection in reaching all TB cases. These are factors common to most high TB endemic countries. According to WHO report 2003, the estimated incidence of all forms of TB in Ethiopia has now reached to 292 per 100000 population (WHO, 2003).

Table 2 Estimate of burden of tuberculosis in Ethiopia, 2001

	ARTI 2.2%	POPULATION: 64,011,875
Incidence Ratio of all forms of TB	260/100,000	166,400 cases
Incidence Ratio of Smear Positive TB	109/100,000	69,760 cases
Prevalence of TB infection	36.0%	23,044,275 persons
Active TB cases co-infected with HIV	30.0%	49,920 cases
TB/HIV co-infection	1.5% of the population	960,178 persons
TB Case Fatality Rate	31%	51,584 cases

1.7 Drug Resistance

1.7.1 Definition

Drug resistance is defined as a decrease in susceptibility of sufficient degree from a wild strain that has never been exposed to the drug (WHO, 1997). Generally, when one percent or more of organisms in an isolate are found resistant to an antituberculous drug, therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Rom and Garay, 1996).

Normally any large population of bacteria, regardless of their exposure to antibiotics will contain some organisms resistant to one of the five first line drugs, isoniazid (H), rifampicin (R), streptomycin (S), ethambutol (E), and pyrazinamide (Z) (Selwyn *et al.*, 1992).

Drug resistance is a natural phenomenon and could occur at any time during bacterial replication. It arises from random mutations of the bacterial chromosome that occur

spontaneously in wild type strains. When mutation confers resistance to a certain antibiotic, all sensitive bacteria are killed and the resistant ones will grow and become the dominant variant in the population (Porter and Adam, 1992).

Although drug resistant strains must have existed before the development of antibiotics their frequency of occurrence increased after the development of anti-tuberculosis drugs in the 1950s (Rom and Garay, 1996).

Drug resistance in tuberculosis could therefore occur in naive patients who have never been treated with any anti tuberculosis drugs i.e. primary resistance or in patients who get inadequate treatment due to prescription error or non-adherence to the appropriate regimen (WHO, 1994).

1.7.2 Molecular Genetic Basis of Drug Resistance

The genetic bases of resistance 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. Rifampicin specifically binds to the β subunit and prevents early steps of transcription that leads to the bacterial death. However, mutation in *rpo B* gene results in resistance by decreasing rifampicin-binding affinity. Mutation leading to resistance of *M. tuberculosis* to rifampicin is rare and occurs at a rate of 10^{-10} per cell division with an estimated prevalence of 1 in 10^8 cells in drug free environment. However, it rapidly results in the selection of mutants that are resistant to other anti-TB drugs (Goble *et al.*, 1993). Most commonly, it exists in conjunction with

isoniazid resistance, and thus defines a strain as being multidrug resistant (MDR) (Turett *et al.*, 1995).

Analysis of rifampicin resistant clinical strains from around the world found that 95 to 98% of resistant strains harbor mutations in an 81 bp hot spot region of the 3534 bp *rpo B* gene (Nachamkin *et al.*, 1997).

Isoniazid is a potent drug that inhibits the synthesis of mycolic acids in *M. tuberculosis*. It is a pro drug that becomes active when catalyzed by the enzyme catalase peroxidase. At least three genes, *kat G*, *inh A* and *ahp C* are involved in resistance to isoniazid. Among these genes, *kat G* that encodes catalase peroxidase is mainly responsible for isoniazid resistance. Approximately 50% of resistant isolates contain mutations in *kat G* with the majority localized to codon 315. It is found that the *kat G* gene encoding catalase peroxidase is defective in many isoniazid resistant strains (Zhang *et al.*, 1992).

A missense mutation of the *inh A* gene which encodes an enzyme involved in the mycolic acid biosynthetic pathway also causes 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 *inh A* mutation alone is rare (Telenti *et al.*, 1997). Mutations have been found in the *ahp C* promoter region of approximately 10% of isoniazid resistant isolates, however these mutations have always been found to occur in association with mutations in *kat G* (Riska *et al.*, 2000). The rate of mutation for isoniazid is 10^{-8} resulting in resistance in 1 out of 10^6 bacilli (Zhang *et al.*, 1992).

Pyrazinamide is also a pro-drug that can be converted into an active form presumably by pyrazinamidase enzyme of susceptible organisms. The target for the active drug is not fully known. However, mutations in the gene *pnc A*, encoding for the enzyme pyrazinamidase are the major causes of pyrazinamide resistance. Between 72% and 98% of pyrazinamide resistance in clinical isolates is correlated with mutations scattered throughout the 558 bp *pnc A* coding region and 11 promoter regions. The rate of mutation for pyrazinamide is 10^{-3} with a probability of resistance 1 out of 10^6 bacilli (Scorpio and Zhang, 1996).

Streptomycin is an antibiotic that interferes with prokaryotic protein synthesis. 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. 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 resistance 1 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 *embCAB* 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).

Multiple drug resistance due to spontaneously occurring mutations is impossible, since there is no single gene involved in MDR and mutations resulting in resistance to various drugs arise independently. Spontaneous mutations resulting in resistance to both isoniazid and rifampicin is the product of the individual probabilities i.e., 1 in 10^{14} ($10^6 \times 10^8$) (Iseman and Madsen, 1989).

A summary of the characteristics of the main anti- tuberculosis drugs is listed in Table 3.

Table 3 Anti- tuberculosis drugs and their characteristics (WHO, 1997)

Drugs	Year of Introduction	Anti- tuberculosis activity	Molecular target	Target gene(s)	Mutation rate	Wild type resistance
Isoniazid	1952	+ + + +	Mycolic acid synthesis	<i>kat G</i> <i>inh A</i> <i>ahp C</i>	10^{-8}	1 in 10^6
Rifampicin	1965	+ + + +	RNA polymerase	<i>rpo B</i>	10^{-10}	1 in 10^8
Pyrazinamide	1970	+ + +	Not known	<i>pnc A</i>	10^{-3}	1 in 10^6
Streptomycin	1944	+ + +	Ribosomal protein	<i>rps L</i> <i>rrs</i>	10^{-8}	1 in 10^7
Ethambutol	1968	+ +	Cell wall polysaccharides	<i>emb A</i> , B & C	10^{-7}	1 in 10^5

1.7.3 Epidemiology of drug resistant tuberculosis

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

This survey collected data from 35 countries of five continents and found MDR tuberculosis to be present in all surveyed countries except Kenya. Drug resistance among new tuberculosis patients was ranged between 2% and 40%. The prevalence of primary MDR had a range of 0 to 14% and a median of 1.4%.

The prevalence of drug resistance varies from drug to drug. Primary drug resistance for isoniazid ranges from 0 to 16.9%, for streptomycin from 0.1 to 23.5%, for rifampicin from 0 to 3%, and for ethambutol from 0 to 4.2%. As expected, the prevalence of acquired drug resistance was higher than primary resistance: for isoniazid it varied between 4% and 53.7%, for streptomycin between 0 and 19.4%, for rifampicin between 0 and 14.5%, and for ethambutol between 0 and 13.7% (WHO, 1997).

The prevalence of resistance to at least one drug, as observed in a recent survey, showed a range between 1.7% and 36.9% among new and from 0% to 14.1% in MDR TB cases (Espinal *et al.*, 2001).

A survey conducted to assess trends in resistance to anti-tuberculosis drugs in 58 countries on six continents showed that MDR TB continues to be a serious problem particularly among some countries of Eastern Europe. In countries with data available for two or more years, there was a statistically significant upward trend in the prevalence of resistance to any drug among new cases (Espinal *et al.*, 2001).

Different reports in Ethiopia have shown that drug resistance is on the increase. Primary MDR-TB was first reported as 2% in samples taken from Addis Ababa and Harar (Wolde

et al., 1986). In 1997, 0.4% of patients from Harar (Mitike *et al.*, 1997) and 1.2% from Addis Ababa (Demissie *et al.*, 1997) were reported to have primary MDR TB. Recently the percentage of MDR in new cases was estimated to be 2.3 (WHO, 2003).

The primary drug resistance profile of *M. tuberculosis* isolates in Addis Ababa indicated that resistance to streptomycin was 10.2%, isoniazid (8.4%), rifampicin (1.8%) and ethambutol (0%) (Demissie *et al.*, 1997).

1.7.4 Diagnosis of Drug resistant Tuberculosis

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

1.7.4.1 Proportion method

The proportion method is based on the principle of calculating the proportion of resistant bacilli present in a strain. High and low dilutions of bacteria are inoculated on drug free and

containing media, in order to provide numerable colonies. The ratio of the number of colonies between the two media indicates the proportion of resistant bacilli present in the strain. Resistance is defined as more growth (above 1%) in drug containing media as compared to drug free control. This method is preferred to others because it does not require standardization of inoculum size (Canetti *et al.*, 1963).

1.7.4.2 Colorimetric assay: MTT assay

MTT assay is a colorimetric that uses a yellow tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) that is converted by dehydrogenase enzyme in living cells to produce insoluble blue formazan crystals. The formazan can then be measured by a spectrophotometer after solubilization (Denizot and Lang, 1986).

The amount of formazan produced is directly proportional to the number of live cells (Mosman, 1983). The same principle can be used to detect the viability of *M. tuberculosis* after exposure to rifampicin. The presence of rifampicin resistance is confirmed when at least 1% of the bacterial populations are composed of drug resistant strains. The assay is cheap, requires less than 4 to 5 weeks and is visually readable (Mshana *et al.*, 1998; Abate *et al* 1998).

1.8 Treatment of tuberculosis

1.8.1 Conventional Treatment

The discovery of streptomycin in 1944 marked the beginning of the era of effective chemotherapy for mycobacterial disease, and the initial clinical trials with isoniazid in 1952 increased the effectiveness of the chemotherapy (Raleigh and James, 1984). At the moment

tuberculosis is effectively treated using combination of the first line drugs that include isoniazid, rifampicin, streptomycin, ethambutol and pyrazinaminamide. Resistance to all of these drugs occurs at high frequency when they are used alone. That is why the drugs are given in two or more combinations (Salyers and whitt, 1994). These drugs are given to TB patients in two phases for at least 6 months. During the initial intensive phase of treatment, which lasts 2 months, the patients are treated with four drugs (H, R, Z, E and S) to ensure that mutants that are resistant to a single drug cannot emerge. In the following four or six months, the continuation phase, two drugs (HR or HE) are given to kill any persisting organisms (Cole, 1994).

The current regimen recommended by WHO for treatment failures consists of (2SHRZE/1HRZE/5HRE) regimen. This regimen is inadequate for cases of tuberculosis resistant to isoniazid and rifampicin (MDR) (Schluger, 2000)

(The numbers before the regimens indicate the duration of therapy, e.g. 2SHRZE indicates a two months regimen with the five drugs)

In Ethiopia, all the five drugs, streptomycin, ethambutol, isoniazid, rifampicin and pyrazinamide are used as anti TB drugs. These drugs are available in loose and fixed dose combination (FDC). The fixed dose combination drugs exist in the form of 2FDC and 3FDC formulations. Ethambutol and isoniazid (EH), rifampicin and isoniazid (RH) exist as 2FDC, where as rifampicin, isoniazid and pyrazinamide (RHZ) are available as 3FDC.

There are three different categories of treatment regimens in Ethiopia and each regimen is recommended for a defined group of patients and their formulation includes the duration of treatment, type of drug and frequency of delivery:

1. Short course chemotherapy (SCC) for smear positive pulmonary tuberculosis (PTB) and seriously ill smear negative PTB and extrapulmonary tuberculosis (EPTB) cases

2S(RHZ)/ 6(EH) or 2E(RHZ)/6(EH)

2. Re-treatment regimen

2SE(RHZ)/1E(RHZ) 5E₃(RH)₃

3. SCC for smear negative PTB, EPTB and TB in children

2(RHZ)/ 6 (EH) (MOH, 2002).

(Intermittent regimens are shown with subscripts, e.g. 5H₃R₃ indicates a three times weekly treatment with two drugs for five months)

1.8.2 Fixed Dose combination Therapy (FDC)

Drug resistance in most tuberculosis patients predominantly arises from multiple interruptions of treatment. When using loose drug formulations, patients are more prone to interrupt their treatment on some drugs while not on others, thereby creating a risk of monotherapy and selection of drug resistant mutants. Furthermore, out of stock or expiry situations in treatment facilities, which might lead to some drugs being continued in isolation while new stocks of others are being awaited, represent another source of monotherapy. Such problems are prevented more easily if fixed dose combinations (FDCs) are used (Blomberg *et al.*, 2001).

FDC is an anti TB drug formulation where two or more anti tuberculosis drugs are prepared in fixed proportions in the same formulation. Two and three drug FDC tablets have been successfully used worldwide since the late 1980's and are registered in more than 40 countries. Now approximately one fourth of the TB cases worldwide receive treatment with rifampicin containing FDC tablets (Norval *et al.*, 1999). This is a strategy adopted to avoid

problems of drug resistance. Therefore WHO and IUATLD recommend the use of FDC in place of loose drugs to:

- Prevent monotherapy and reduce the emergence of drug resistant tuberculosis.
- Simplify treatment by minimizing prescription error, increase patients and doctors compliance. Unlike the conventional treatment patients have to take a large number of tablets, usually 9-16 per day for two months at the initial phase followed by 3-9 tablets daily for 4-6months at continuation phase. In FDCs, the number of tablets to be taken can be reduced to as few as three or four per day for the whole course of treatment.
- Simplify drug stock management, shipping and distribution
- Reduce the risk of misuse of rifampicin for conditions other than tuberculosis (WHO, 1999).

Drug quality is important for all anti tuberculosis drugs but there are particular concerns regarding the bioavailability of rifampicin in FDCs. Bioavailability refers to the amount of drug, which gets absorbed into the blood. It is known that unless rifampicin is combined in optimal proportion with other drugs in the same formulation, its bioavailability is negatively affected. Giving FDC tablets with poor rifampicin bioavailability would mean inadequate therapy, without being aware of it. This could directly lead to poor treatment outcome and drug resistance (Blomberg *et al.*, 2001).

In Ethiopia TB patients have been treated with three fixed dose combination therapy (3FDC) starting from 1999. The individual RHZ tablet strength is R150mg + H75mg + Z400mg. The number of FDC tablets administered depends on the age and weight of the

patients. Previously treated adolescents and adults for example take from 1 to 4 tablets daily during the intensive phase of treatment depending on their body weight. Patients with 20-29kg pre-treatment weight take 1 tablet where as those with 30-37kg, 38-54kg and above 55kg body weight take 2, 3 and 4 tablets respectively (MOH, 2002).

1.9 Re-treatment cases

Re-treatment cases are defined as those cases, who have already received at least one month of anti- tuberculosis therapy. These cases include defaulters, failures, relapses and chronic cases (WHO, 2000a).

Defaulter is a patient who has been on treatment for at least four weeks and whose treatment was interrupted for more than eight consecutive weeks or for a cumulative period of more than twelve weeks

Failure is a patient who remained smear positive at the end of the five months or later, after commencing treatment.

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

Chronic case is patient who is still smear positive at the completion of re-treatment regimen (MOH, 2002).

Worldwide the prevalence of drug resistance among re-treatment cases is quite alarming.

The second report of global project (1996-1999) showed that among previously treated cases in 47 geographical settings resistance to at least one drug ranged from 0% to 94%

(median=26.9%). The prevalence of MDR TB ranged from 0% in 4 geographical settings to 41.7% (median=11.1%) (WHO, 2000a).

The number of re-treatment cases in Ethiopia is on the increase (Figure 1). Parallel to an increment in number, a high rate of drug resistance is also observed. Annual statistical data from St Peter's specialized TB Hospital showed that the number of previously treated patients increased from 2% in 1998 to 6% in 2000 (Dr Solomon Goshu, Personal communication, 2002).

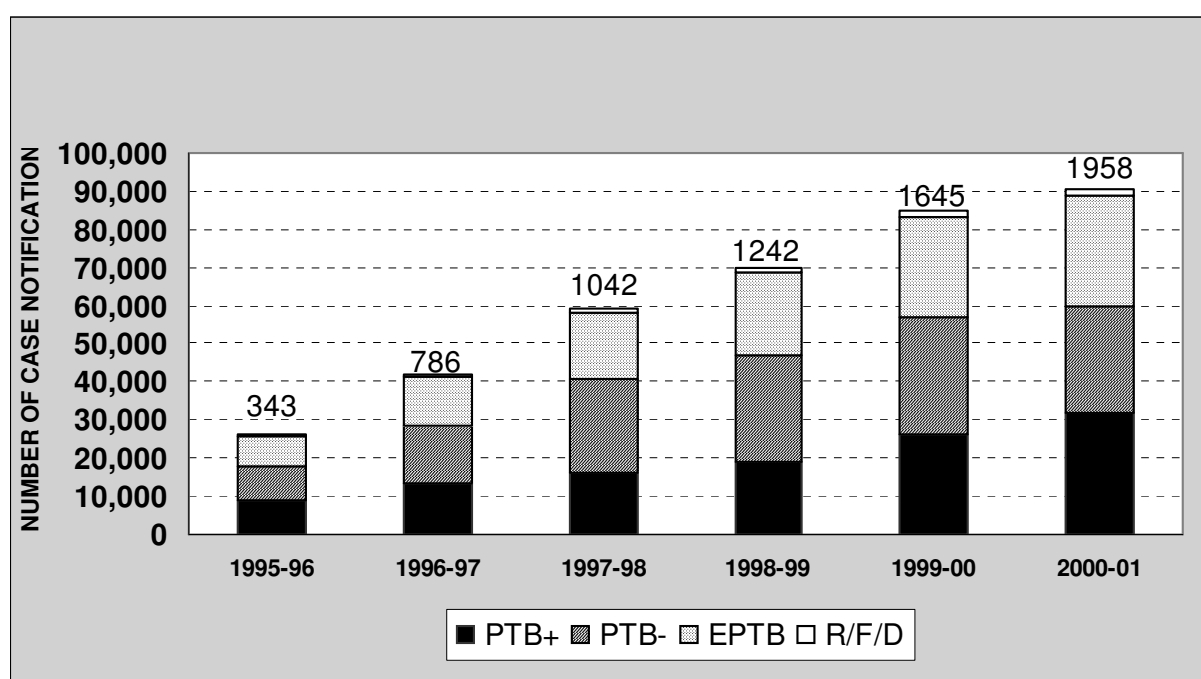


Figure 1 A 6-year overview of TB case notifications by form of TB (data from DOTS areas). The numbers on top of each bar shows re-treatment cases in each year (Source: TLCT, 2001)

A drug resistance survey on re-treatment cases showed that about 50% of the strains were resistant to one or more of the first line drugs and 12% MDR. The same study on re-

treatment cases in Addis Ababa indicated that drug resistance for isoniazid is 44%, streptomycin (28%), rifampicin (12%) and ethambutol (2%) (Abate *et al.*, 1997).

The analyses of laboratory record of 1100 drug susceptibility tests performed between 1994-1999 on treatment failure and relapse cases showed 17.6% multidrug resistant tuberculosis. Currently, multi-drug resistant strains look to originate largely from patients who are receiving re-treatment (TLCP, 2002).

1.10 Characterization and identification of *M. tuberculosis* strains

1.10.1 Genetic characterization

The most commonly used method of genetic characterization of strains is a restriction fragment length polymorphisms pattern analysis (also called molecular fingerprinting). This method is based on differences in the 1355bp insertion sequence (IS) 6110 copy numbers per strain, ranging between zero and 25 and variability in the chromosomal positions. Insertion sequences are small mobile genetic elements that encode their own transposition (Hermans *et al.*, 1990).

In this technique, DNA is extracted and purified from bacterial culture. Thereafter, the DNA is digested with the restriction enzyme *pvu* II and the restriction fragments are separated on an agarose gel. The separated restriction fragments are transferred to a DNA membrane. In order to visualize the IS6110 containing restriction fragments, a peroxidase labeled horseradish enzyme probe with a DNA sequence complementary to the IS 6110 DNA sequence is added to a hybridization buffer, which is poured onto the membrane. The 245bp IS6110 probe hybridizes to the *pvu* II restriction fragments and this can be enhanced

by a chemiluminescence reaction initiated by adding two substrates (hydrogen peroxide and luminol). The membrane is packed in plastic and the RFLP patterns are detected by putting a light sensitive film on the packed membrane in a light blocked cassette (van Soolingen, 2001).

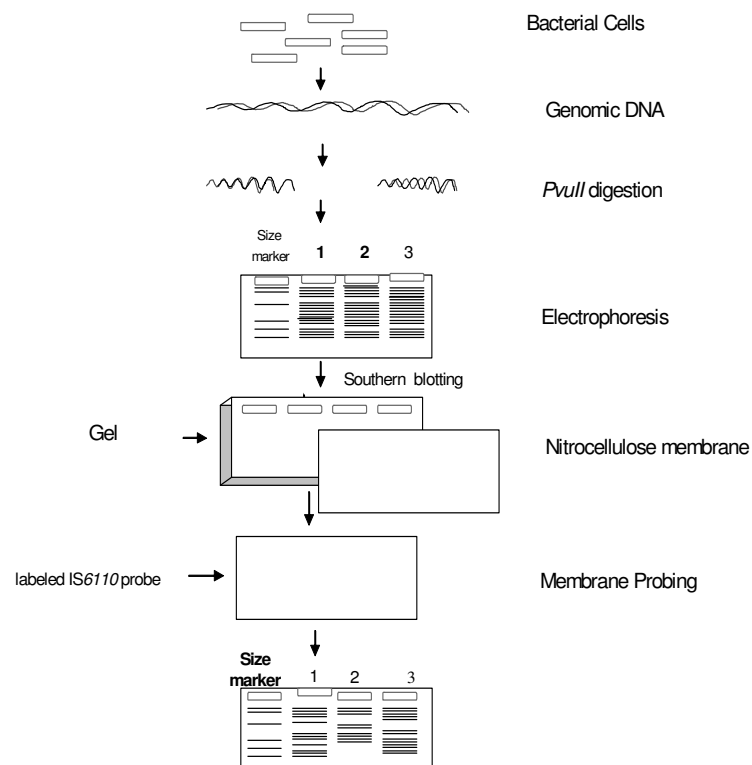


Figure 2 The process of RFLP analysis

RFLP characterization is important for:

1. Routine control of tuberculosis

With an effective control program, one does not expect *M. tuberculosis* isolates with identical or highly similar DNA fingerprints. Demonstration of fingerprints indicates recent transmission, and a failure of control programs. Identifying clusters and then sites of transmission therefore helps to focus control efforts.

2. Geographic tracking of *M. tuberculosis* strains

This enables tracking of strains over broad geographic areas. DNA fingerprint analysis is used to determine the factors, which promote transmission between distant or adjacent regions.

3. Identification of *M. tuberculosis* strains with unique phenotypic characteristics

This could be utilized to identify strains with particular properties, such as high infectivity, virulence, and/or multidrug resistance (van Embden *et al.*, 1993).

1.10.2 Identification of *M. tuberculosis* (Biochemical Tests)

After a mycobacterial isolates have been classified to a subgroup on the basis of pigment production, colonial morphology and growth rate, specific identification is accomplished using different biochemical tests. Final identification is based on more than one test because individual strains may deviate from the expected results. These tests include nitrate reduction, pyrazinamidase and thiophene 2 carboxylic acid hydrazide tests.

1.10.2.1 Nitrate Reduction Test

Mycobacteria differ quantitatively in their abilities to reduce nitrate and the test measures the ability of a strain to produce the enzyme nitrate reductase. It is valuable for the identification of some mycobacteria that possess similar characteristics of colony

morphology, growth rate and pigmentation. *M. tuberculosis*, *M. kansasii*, *M. szulgai* and most rapid growers are nitrate reductase positive (Virtanen, 1960).

1.10.2.2 Thiophene 2 carboxylic acid hydrazide (TCH) test

TCH test is used to distinguish niacin positive *M. bovis* from *M. tuberculosis* and other non-chromogenic slowly growing mycobacteria. *M. bovis* is susceptible to low concentrations (1 to 5 µg/ml) of TCH where as *M. tuberculosis* and other mycobacteria are resistant to the inhibitory action of this compound (Vestal and Kubica, 1967).

1.10.2.3 Pyrazinamidase Test

The enzyme pyrazinamidase hydrolyses pyrazinamide to pyrazinoic acid. This acid is detected by the addition of ferrous ammonium sulfate to the culture medium. The formation of a pink ferrous- pyrazinoic acid complex indicates a positive test. This test is most useful in separating *M. tuberculosis* from *M. bovis* (Wayne, 1974).

1.11 Scientific Problem

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, and occasionally by *M. bovis* and by *M. africanum*, which are the main pathogenic species with in the *M. tuberculosis* complex (WHO, 2000b). It has afflicted humanity for over thousands of years, and probably accounts for more human deaths than any other single pathogen (Cole, 1994).

Although the causative agent of the disease was first detected in 1882 by the German scientist Robert Koch, prehistoric arts and different reports of spinal tuberculosis in skeletal remains witness that tuberculosis was a health problem as early as 4000 BC (Morse, 1961).

Anti-tuberculosis drugs such as rifampicin, isoniazid, ethambutol, streptomycin, pyrazinamide and BCG vaccine have been used to fight the disease. However, after decades of decline, tuberculosis is emerging associated with human immunodeficiency virus (HIV) and resistance to the current anti-tuberculosis drugs (van Soolingen, 2001).

This increase in drug resistant tuberculosis in Africa, Asia and Latin America is very dramatic. The main reason for the emergence of drug resistant strains is a result of insufficient or inappropriate chemotherapy associated with either non-compliance by the patient or administration of inadequate drug regimens (Mitchison, 1998).

Today drug resistant TB poses a great health risk to individuals as well as to communities. In order to solve this problem a new strategy of treatment has been developed. That is loose drug treatment is replaced by fixed dose combination (FDC) therapy. FDCs are expected to simplify treatment, increase patient and doctor compliance, simplify drug supply management and reduce misuse of rifampicin for conditions other than TB. However when low quality FDC drugs are used, treatment failure and emergence of drug resistance could occur (Blomberg *et al.*, 2001).

Therefore, it is important to monitor drug sensitivity patterns as a part of a national TB control program. Understanding drug resistance patterns in a community has enormous epidemiological significance, which are useful in evaluating the quality of the anti TB

treatment provided to the community (Chaulet *et al.*, 1995). To this effect rapid and cheap methods of diagnosing drug resistance strains are required and these will also help to provide proper treatment particularly to individual patients harboring resistant strains like multi-drug resistant strains (MDR). Based on the above problems the following objectives are set:

1.12 Objectives

1.12.1 General objective

To assess the drug resistance pattern among tuberculosis patients of re-treatment cases after introduction of three fixed dose combination (3FDC) therapy.

1.12.2 Specific objectives

- 1.12.2.1 To study the prevalence of drug resistance in isolates of *M. tuberculosis* from re-treatment cases of pulmonary TB in St Peter's TB specialized Hospital
- 1.12.2.2 To compare the drug resistance profile of patients treated with loose and three fixed dose combination regimen
- 1.12.2.3 To further develop and evaluate the colorimetric assay (MTT) for direct detection of rifampicin resistance.
- 1.12.2.4 To characterize *M. tuberculosis* isolates using insertion sequence, IS6110 restriction fragment length polymorphisms (RFLP) pattern.

2. Materials and methods

2.1 Study population

All subjects were selected from those patients who came to St Peter's TB Specialized hospital for treatment between 21 December 2001 and 15 October 2002. They were all re-treatment cases with smear positive sputum. The sputum samples were collected before initiation of re-treatment from defaulters, chronic cases, failures and cases with relapse (Definition, PP 17). The attending physician screened the patients and filled the clinical form (Appendix-1).

The sample size was determined based on the prevalence of rifampicin resistance (12%) in a previous study on re-treatment cases in Addis Ababa. With 12% prevalence the required sample size was 117. However, the limited time and number of patients coming to the hospital during the study period allowed us to collect only 100 sputum samples.

2.2 Sputum specimen

A single sputum specimen from each patient was collected in a 20 ml screw capped universal tube from 100 smear positive re-treatment cases and transported to Armauer Hansen Research Institute (AHRI) laboratory. The samples were kept at 4°C and processed within two days.

2.3 Bacterial culture

The sputum was digested and decontaminated using Petroff's method. Equal volume of 4% NaOH was added to the sputum in 50ml screw capped bottle. After vigorous vortex the sample was centrifuged for 15 minutes at 1620g. One drop of phenol red indicator was

added before neutralization by 2N HCL. A change from red to yellow color indicates neutralization. The pellets were resuspended in 2.5ml PBS. An aliquot of 100µl of the sample was then cultured into two tubes containing Lowenstein Jensen (LJ) media for primary isolation. The tubes were incubated at 37⁰C and examined for bacterial growth until week eight. From the remaining resuspended sample, 500µl was inoculated into each of the 4 tubes containing 3ml Middle brook 7H9 broth supplemented with Oleic acid albumin dextrose catalase (OADC) enrichment (Beckton-Dickinson) and antibiotics mixture (PANTA) (Becton-Dickinson). PANTA is a standard antibiotic cocktail that contains Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin. One of the four tubes contained rifampicin at 2mg/l final concentration (Sigma) while the rest were drug free controls. They were then incubated at 37⁰C and MTT assay was done on week one, two or three depending on the formation of formazan in the control tubes.

2.4 Susceptibility to anti-tuberculosis drugs

2.4.1 Preparation of drug solutions

Stock solutions were prepared for each drug in phosphate buffered saline (PBS) at different concentrations. After filter sterilization using 0.2µM filter, aliquots of the drug solutions were kept in cryotubes and stored at –70⁰C (Table 4).

Two stocks of drug solution were prepared using isoniazid. Stock one was prepared at a concentration of 1mg/ml in 20ml of PBS and added into the medium to get a final concentration of 1mg/l. This drug solution was diluted to prepare stock two, which has a concentration of 0.2mg/ml. A final concentration of 0.2mg/l was obtained after the addition of stock two into the medium.

One stock solution was prepared for rifampicin. About 20mg of the drug was first dissolved into 2ml of concentrated ethanol. Adding 18ml of PBS then diluted the drug solution, which was then added into the medium to get a final concentration of 1mg/l.

Streptomycin (stock one) was prepared by dissolving 200mg of the drug in 20ml PBS. This drug solution was added into the medium to get a final concentration of 10mg/l. Stock two with a concentration of 2.5mg/ml was prepared from stock one and added to the medium to get a final concentration of 5mg/l.

Similarly stock one of ethambutol was prepared by dissolving 200mg of the drug in 20ml PBS. This drug solution was added into the medium to get a final concentration of 10mg/l. Stock two with a concentration of 6.25mg/ml was prepared from stock one and added into the medium to get a final concentration of 5mg/l.

A stock solution of 3- (4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) (Sigma) at a concentration of 5mg/ml was prepared in PBS and was kept at 4⁰C in the dark. A final concentration of 0.5mg/l was used in the assay.

Table 4 The concentration and solvents of anti-tuberculosis drugs (Source , Inderlied and Salfinger, 1995)

Drugs	Product number (Sigma)	Solvent(s) used	Final concentration		
			DST *		MTT assay
			Low	High	
Rifampicin	R-3501	Ethanol/ PBS	NA	1mg/l	2mg/l
Isoniazid	I-3377	PBS	0.2mg/l	1mg/l	NA
Streptomycin	S-6501	PBS	2mg/l	10mg/l	NA
Ethambutol	E-4630	PBS	5mg/l	10mg/l	NA

NA Not applicable * Modified proportion method

2.4.2 Drug sensitivity test (DST)

Standard indirect drug susceptibility test was done to isoniazid, rifampicin, streptomycin and ethambutol. The test was done based on the modified proportion method (Canetti *et al.*, 1969) using Middle brook 7H10 supplemented with 10% OADC and glycerol. A bacterial suspension with turbidity corresponding to McFarland turbidity standard-1 (OD value between 0.25 and 0.30 at 600 nm using (Novaspec II photometer, Pharmacia Biotech Ltd, UK) was prepared in two dilutions as 1: 10 and 1:1000. From the 1:10 bacterial dilution, 100µl was transferred into all drug containing tubes and one drug free control tube. About 100µl of bacterial suspension from the 1:1000 dilution was transferred into one drug free control tube. All the tubes were then incubated at 37⁰C and examined for colony formation every week until the third week. One reference strain (ATCC 35836, streptomycin resistant or ATCC 35838, rifampicin resistant) was included in each test batch as a control. The proportion of growth was calculated by dividing the number of colonies in a drug medium with the drug free medium when the numbers of colonies on drug free media were 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. We could also compare the number of colonies in a drug containing tube inoculated with 1:10 dilution with a control containing 1:1000 diluted bacterial suspension. A higher number of colonies in a drug containing media indicated resistance.

2.4.3 MTT assay

MTT is a yellow tetrazolium salt that is converted into a blue formazan by dehydrogenases of a live cell. The assay is based on the principle that the amount of formazan produced is directly proportional to the number of live cells (Mosmann, 1983). The assay was done by

adding 300µl of 5mg/ml MTT solution into the 7H9 broth medium and dissolving the formazan produced with solubilization buffer (20% sodium dodecyl sulfate (SDS) in a 50% aqueous solution of dimethyl formamide (DMF) after 4hrs of incubation at 37⁰C (Mshana *et al.*, 1998). Optical density (OD) at 570nm (Novaspec II photometer, Pharmacia Biotech Ltd, UK) was then measured after 1hr of incubation at 37⁰C, using a reference tube containing 7H9 broth, PBS, MTT and solubilization buffer. Relative optical density unit (RODU) values were calculated by dividing the OD of the drug containing tubes with the OD of drug free control. The MTT assay was done on the day after a visible formazan was observed in the control medium. However, when no formazan was seen on the first and second week of incubation, the assay would be performed in the third week.

A strain was defined as rifampicin- susceptible when the relative optical density unit (RODU) was 0.2 and resistant when the RODU was more than 0.5.

After a thorough vortex, 100µl of the bacterial suspension was transferred from the 7H9 medium to the tubes containing 7H10 agar before doing the MTT assay. The tubes were incubated at 37⁰C and observed each week for colony forming unit (CFU). The OD value from the MTT result was correlated with CFU to determine the lowest OD value that corresponds to the CFU. The result was considered uninterpretable if the optical density of the control remained below 0.1 through the third week. A test for bacterial contamination was performed on nutrient agar before performing the MTT assay.

2.5 Characterization of *M. tuberculosis* strains

2.5.1 Restriction Fragment Length PolymorphiS (RFLP)

2.5.1.1 DNA extraction

The standard protocol for nucleic acid extraction of Mycobacteria was followed (van Soolingen *et al.*, 1999) to extract DNA from *M. tuberculosis* isolates grown on LJ medium. One loop full of *M. tuberculosis* cells was transferred into a 1.5ml microcentrifuge tube containing 400µl of 1x Tris-Ethylenediamine tetra acetic acid (EDTA) (TE) buffer. The cells killed by heating for 20 min at 80°C were centrifuged for 5 minutes at 12000rpm. About 50 µl of 10mg/ml lysozyme (Sigma, Saint Louis, Mo) was added into the bacterial suspension and incubated for overnight at 37°C. This was followed by addition of 70 µl of 10% sodium dodecyl sulfate (SDS) (Sigma) and 6 µl of 10mg/ml Proteinase K (GIBCO/BRL-Life Technologies, Gaithersburg, Md) and incubation at 65°C for 10minutes. Proteinase K removes protein from the nucleic acid while SDS facilitates their dissociation. Cell wall debris, denatured protein and polysaccharides form complex with Cetyltrimethyl Ammonium Bromide (CTAB) when 100 µl of 5M NaCl and 80µl CTAB/ NaCl solution was added and vortexed until the solution become "milky". After incubation for another 10 minutes at 65°C the nucleic acid was extracted using 700 µl of chloroform/ isoamyl alcohol (24:1). Precipitation of the nucleic acid was done by adding 0.6 volume of isopropanol and keeping it at -20°C for 30 minutes. The DNA was collected after spinning for 15 minutes at room temperature at 12000 rpm. The pellet was washed with 1 ml cold 70% ethanol to remove residual CTAB and NaCl. Finally the pellet was redissolved with TE buffer and stored at -20°C until further use.

2.5.1.2 Preparation of probe

The IS6110 probe was prepared from a 245bp DNA fragment amplified by the polymerase chain reaction (PCR) from a TB strain Mt14323. The primers used to amplify the region were: -

INS-1 (5' CGTGAGGGCATCGAGGTGGC)

INS-2 (5' GCGTAGGCGTCGGTGACAAA)

The IS6110 region was amplified using a PCR program:

1 cycle of 3 min at 96⁰C, 40 cycles of 40 sec at 94⁰C, 40 sec at 65⁰C and 15 sec at 72⁰C in a thermo- cycler (Thermo Hybaid, UK).

The probe was labeled using the enhanced chemiluminescence gene detection system (ECL) (Amersham Pharmacia Biotech UK Limited 2001). The system involved direct labeling of single stranded DNA with the horseradish peroxidase enzyme. This was achieved by completely denaturing the DNA to single strand. When peroxidase, which had been complexed with a positively charged polymer is added it forms a loose attachment to the nucleic acid by charge attraction. Addition of glutaraldehyde causes the formation of covalent cross-links thereby producing enzyme labeled probe (van Soolingen, 1999).

2.5.1.3 RFLP analysis

DNA fingerprinting was performed as described by van Embden *et al.*, 1993. After extraction of the mycobacterial DNA it was digested by *pvu* II restriction enzyme (Biolab, England). About 4.5 kg of the DNA samples was digested with 1µl, *Pvu* II (10,000 u/ml) in a final volume of 20µl. The mixture of genomic DNA, 1 X NEB buffer 2, *Pvu* II and distilled water was incubated for 2 hours at 37⁰C.

An agarose gel (0.8 %) was then prepared using 1xTBE buffer. About 12-20µl of digested product mixed with 5X sample buffer and internal marker (Super coiled DNA ladder digested with *Pvu II* and phi X-174 digested with Hae III) was applied into each well in the gel.

An electrophoresis marker, Hind III digested Lambda DNA was loaded in the first and *M. tuberculosis* reference strain Mt 14323 in the last well as an external marker. The gel was then subjected to electrophoresis at 100 volts for 10 min and then at 20 volts over night. The DNA in the gel was exposed to UV light for 5 min and treated with 0.25M HCL to break it into smaller fragments and make an efficient transfer. This was followed by denaturation of DNA fragments using 0.4 M NaOH.

The digested DNA from the gel was then transferred to a hybond N+ positively charged nylon membrane by capillary method using the transfer buffer (10 X SSPE). After washing, the membrane was hybridized overnight at 42 °C with the IS6110 probe. The IS6110 bands were then detected using hydrogen peroxide and luminol (supplied with ECL kit, Amersham International, Amersham, UK) to be observed on the autoradiograph.

2.5.2 Biochemical test

The following tests were done on randomly selected isolates (Kent and Kubica, 1985).

2.5.2.1 Nitrate reduction test

The ability of *M. tuberculosis* to reduce nitrate is valuable for identification of the species. Most mycobacterial cultures to be tested for nitrate reduction should be examined 4 weeks after inoculation onto the subculture medium. About 0.2 ml of sterile saline was added to the culture followed by 2 ml of the NaNO₃ substrate. After shaking it well, the tube was incubated for 2 hrs at 37°C. One drop of 4HCl, 2 drops of sulfanilamide and 2 drops of

Napththylethylene diamine dihydrochloride were then added in order. A change from pink to red color confirms a positive result.

The absence of color indicates either negative or a reduction reaction beyond nitrite. Powdered zinc is added to all tubes with no color and red color indicates a true negative while no color confirms a positive result.

2.5.2.2 Thiophene 2 carboxylic acid hydrazide (TCH) test

Two tubes of OADC enriched Middlebrook 7H10 medium were prepared. Filter sterilized TCH was added in one of the tubes to make a final concentration of 2µg/ml. The drug containing and drug free tubes were inoculated with 100µl of 10⁻¹ diluted bacterial suspension with a turbidity corresponding to the McFarland turbidity standard-1 and incubated for three weeks. *M. bovis* strain was used as a positive control in every batch. 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.

2.5.2.3 Pyrazinamidase test

Two tubes with the substrate medium (Dubos broth, Pyrazinamide, Pyruvic acid, Sodium salt and agar) were inoculated with a heavy loop full of bacteria from an actively growing 2 to 3 weeks old culture. They were then treated with freshly prepared 1% ferrous ammonium sulfate solution for 30min at room temperature after incubation at 37⁰C for four days and seven days. A pink band on the surface agar confirms the presence of *M. tuberculosis*. An *M. bovis* containing medium treated similarly was used as a negative control.

2.6 Statistical Analysis

Statistical analysis was carried out using Stata version 7 software. Odds Ratio (OR) and 95% confidence intervals (CI) were calculated. Chi square test was done to compare drug resistance prevalence in different groups. A *P* value < 0.05 was considered statistically significant.

2.7 Ethical Consideration

This project has obtained ethical clearance from Ethiopian Science and Technology Commission under a project entitled "Standardization of the direct MTT assay for the detection of rifampicin resistance". Informed consent was obtained from all patients before taking the sputum samples.

3. RESULT

3.1 Study population

3.1.1 General characteristics

A total of 100 patients were considered for investigation. All were previously treated sputum smear positive tuberculosis cases. The age as shown in Table 5 ranges from 16 to 65 and the median age was 30 years. Out of 100 patients 70 were males.

Table 5 Distribution of patients by age and sex

Sex	Number of patients in different age groups (Age in years)						Total
	15-24	25-34	35-44	45-54	55-64	65+	
Male	14	27	18	10	1	0	70
Female	7	15	5	2	0	1	30
Total	21	42	23	12	1	1	100

The majority of the patients 56% and 32% were from Addis Ababa and places around Addis Ababa respectively (Table 6). As shown in Table 6 these patients had previously taken loose and fixed dose combination regimens. Thus, 53 of them had a history of treatment with 3FDC regimen during the intensive phase of treatment and the remaining 47 had taken loose drugs.

Table 6 Number of patients from different places and previously used drug regimen

Place	Type of Regimen		Total
	3FDC	Loose	
Addis Ababa	31	25	56
Around A.A	16	16	32
Awassa	1	0	1
Gojjam	1	1	2
Harar	1	0	1
Jimma	0	1	1
Chencha	0	2	2
Wuchale	1	0	1
Yirgalem	1	0	1
No address	1	2	3
Total	53	47	100

3.1.2 Medical history

There were four types of patients: sixty patients were cases with relapse, 15 were defaulters, 13 failures and 12 chronic cases. The distribution of types of patients by age is shown in Table 7.

Table 7 Distribution of patients by type and age group

Age group	Patient type				Total
	Chronic	Defaulter	Failure	Relapse	
15-24	4	1	2	14	21
25-34	3	5	7	27	42
35-44	3	7	2	11	23
45-54	2	2	1	7	12
55-64	0	0	1	0	1
65+	0	0	0	1	1
TOTAL	12	15	13	60	100

The remission period of the patients (a period that the patient remains free from symptoms after the last treatment) ranges from 0 to 29 years with a mean of 3.3 years and a median of 1.9 year.

The clinical history of the patients showed that all of them had productive cough at least for three weeks. A total of 94 patients said that they had fever, 38 patients' haemoptysis, 63 night sweat and 40 chest pain.

The current x ray findings showed that 65% of them had right or left infiltration or opacification, 22% bilateral infiltration and 13% had cavity in their lungs. From the 44 patients with one or more drug resistant isolates 70.7% (52/75) of them had right or left infiltration or opacification, 16% (12/75) bilateral infiltration and 13.3% (10/75) were with cavity in their lungs. Among the 44 patients with one or more drug resistant isolates 75% (33/44) had infiltration in one of their lungs, 15.9% (7/44) had cavity and 9.1% (4/44) had bilateral infiltration. All the 7 patients with cavity in their lungs were MDR cases.

3.2 Culture

A total of one hundred smear positive sputum samples were collected from an equal number of patients. Eighty-nine samples were found out to be culture positive and eleven were culture negative.

3.3 Biochemical test

Nitrate reductase and pyrazinamidase tests were done on 20 randomly selected isolates of *M. tuberculosis* and all of them were found positive. All the 57 isolates tested for TCH susceptibility were resistant to 2mg/l TCH.

3.4 Susceptibility to first line drugs

Drug sensitivity test was done only for 75% of isolates. These isolates were collected from different types of patients. Thus, 64% (48/75) were from patients with relapse case, 13.3%(10/75) from defaulters, 13.3%(10/75) from chronic cases and 9.3%(7/75) from failures.

The highest rate of drug resistance was observed in patients having an age between 25 and 34. The pattern of drug resistance was shown in Figure 3 and resistance to first line drugs was not significantly related to age groups ($P = 0.90$) and gender ($P = 0.45$). The proportion of males (72.7%) among drug resistant cases was higher compared to the females

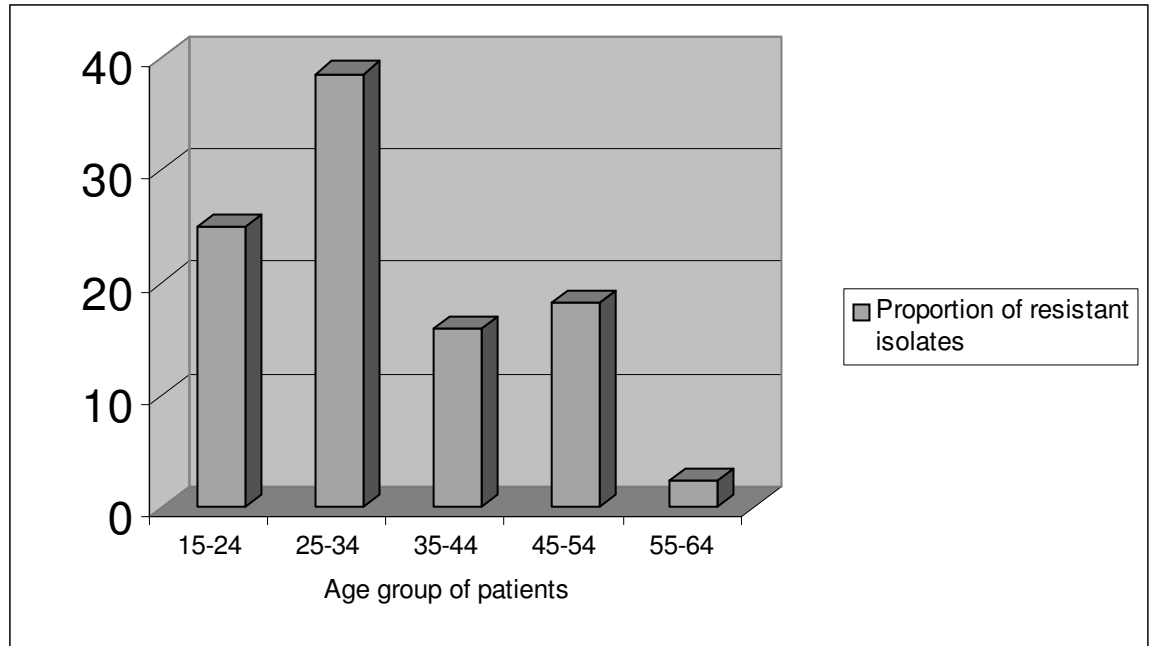


Figure 3 Proportion of drug resistant isolates by age group

The prevalence of drug resistance shows an upward trend particularly to rifampicin and ethambutol. The highest rate of resistance is observed against isoniazid while the lowest is to ethambutol. As it is shown in Figure 4, 42.7% isolates were resistant and 6.7% partially resistant to isoniazid. Resistance to rifampicin was found to be 33.3% where as to streptomycin it is 21.3% resistance and 12% partially resistance. The lowest rate of resistance was observed against ethambutol where 9.3% isolates were resistant and 25.3% partially resistant. Resistance to only one drug (monoresistance) was observed in 12% (9/75) patients with the highest rate against isoniazid (8%). Among all the 75 isolates, resistant to at least one drug was observed in 58.7% (44/75) isolates.

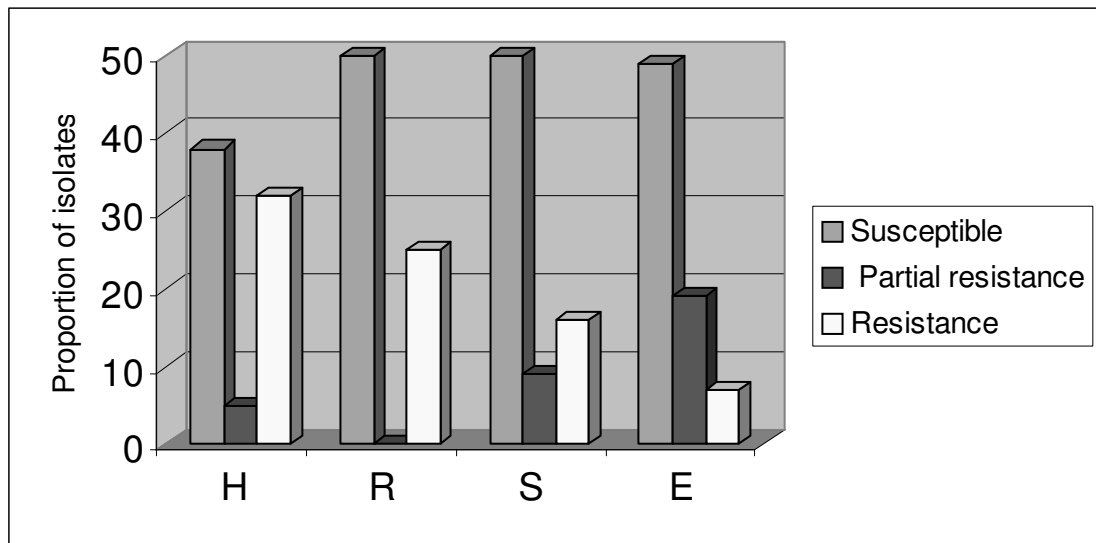


Figure 4 Drug resistance patterns of *M. tuberculosis* isolates to each of the four first line anti- tuberculosis drugs

Resistance to isoniazid and rifampicin (MDR) was observed in 21 patients (28%). As shown in Table 8, a high rate of MDR cases was observed in failures and chronic cases.

About 56.8% of the 44 patients harboring drug resistant strains were relapse cases, 12% chronic cases, 15.9% failures and 6.8% defaulters. A high prevalence of resistance was observed in chronic cases and failures (Table 8).

Table 8 Proportion of resistant isolates among each category of patients.

Type of Patient	Number tested	Isolates fully susceptible (%)	Isolates resistant to one or more drugs (%)	MDR cases (%) *
Relapse	48	23 (48)	25 (52)	8 (16.6)
Chronic cases	10	1 (10)	9 (90)	8 (80)
Defaulter	10	7 (70)	3 (30)	0 (0)
Failure	7	0 (0)	7 (100)	6 (85.7)
Total	75	31 (41.3)	44 (58.7)	22 (29.3)

* Isolates resistant to rifampicin and isoniazid in each category of patients

As shown in table 9 resistance to two drugs had been observed in 17.3% (13/75), to three drugs in 13.3 % (10/75) and to four drugs in 16% (12/75) patients. Relatively a high number of patients had isolates resistant to two drugs.

Table 9 *M. tuberculosis* strains resistant to combination of first line drugs (n=44)

Drugs	Number of resistant strains (%)
H + R	2
H + S	4
H + E	3
R + E	3
S + E	1
Total	13 (17.3)
H + R + S	5
H + R + E	3
H + S + E	2
Total	10 (13.3)
H + R + S + E	12 (16)

The drug resistance pattern of 75 isolates was assessed based on patients who had prior treatment with 3 FDC and those who were treated with loose drugs. As it was shown in table 10, 53.3% (40/75) of the patients had a history of treatment with 3FDC regimen. Analysis of the result showed that there is no statistically significant difference in the development of resistance to one or more drugs between patients who were treated with 3FDC regimen and loose drugs ($P= 0.09$).

From the 40 patients who had prior treatment with 3FDC regimen, resistance to one or more drugs was observed in 27 patients. From the 35 patients who were treated with loose drugs 17 patients had resistance to one or more anti- tuberculosis drugs.

Resistance to rifampicin and isoniazid in patients with prior 3FDC and loose drug treatment was assessed to compare the efficacy of the two regimens. As it was indicated in Table 10 out of the 40 patients who had been treated with 3FDC regimen 25 patients had isolates resistant to isoniazid. The rate of isoniazid resistance was significantly higher in patients who had a history of treatment with 3FDC than in those treated with loose drugs ($P=0.02$).

Of the 40 patients who had a history of treatment with 3FDC regimen, drug resistance to rifampicin was observed in 19 patients (Table 10). From the total number of patients who had prior treatment with loose drugs, 6 patients had rifampicin resistant *M. tuberculosis* isolates. The rate of rifampicin resistance was significantly higher in patients who had a history of treatment with 3FDC regimen than in those treated with loose drugs ($P = 0.005$).

Table 10 Prevalence of rifampicin and isoniazid resistance in patients treated with 3FDC regimen and loose drugs.

Drug Regimen	Rifampicin		Isoniazid	
	Resistance	Susceptible	Resistance	Susceptible
3FDC	19	21	25	15
Loose	6	29	12	23
Total	25	50	37	38

3.5 Direct susceptibility test by MTT assay

When 75 isolates were directly tested for rifampicin resistance using MTT method. The OD value that gives interpretable result was determined by transferring 100µl of the bacterial suspension from the growth medium (7H9) into 7H10 agar to observe colony-forming units. This was done before doing the MTT assay. As shown in figure 5, the cut off OD value to determine the result as interpretable was 0.1.

Based on the cut off value all results with OD value of the control below 0.1 were excluded.

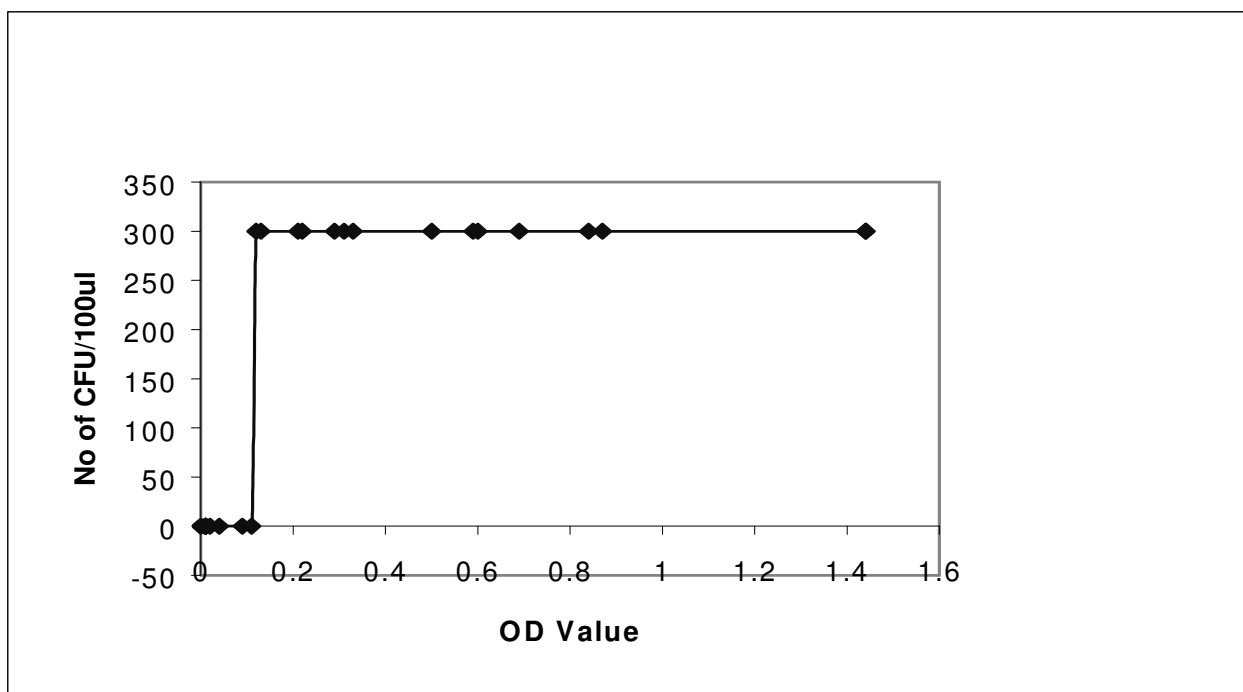


Figure 5 Correlation of OD value with colony forming units for the determination of interpretable results

The assay identified 30.7% of the isolates as resistant and 69.3% as susceptible. The result of the MTT assay matched 97.3% with the proportion method. Two isolates out of the 75 were observed as having discordant result.

Relative optical density units (RODU) is used to define resistance and susceptible strains. There may be strain differences in growth characteristics between rifampicin sensitive and resistant strains and this could account for the variations observed between isolates in the ability to reduce MTT. Therefore a relative value, RODU is used to equalize strain differences in the ability to reduce MTT and emphasize the pattern obtained (Mshana *et al.*, 1998).

The mean RODU value of resistant strains was 1.08 ± 0.5 and it was above 0.5 for all the resistant strains. The mean RODU value of susceptible strains was 0.04 ± 0.06 . Except for one susceptible isolate with a value of 0.3 all had a RODU value below 0.2. This result was in match with the RODU values for resistant and susceptible strains in standardized indirect MTT assay (Abate *et al.*, 1998; Foongladda *et al.*, 2002; Caviedes *et al.*, 2002).

About 19.2% of isolates gave interpretable result at week one, 67.9% at week two and 100% at week three. All rifampicin susceptible and rifampicin resistant strains were correctly identified with the naked eye.

3.6 RFLP analysis

The mycobacterial IS probe was prepared by horseradish peroxidase labeling of a 245 bp fragment PCR product. The two primers, INS-1 and INS-2 were used to amplify the 245-bp DNA fragment (Figure 6) from *M. tuberculosis* reference strain Mt 14323. The PCR product was then purified using MicroSpin Columns and labeled.

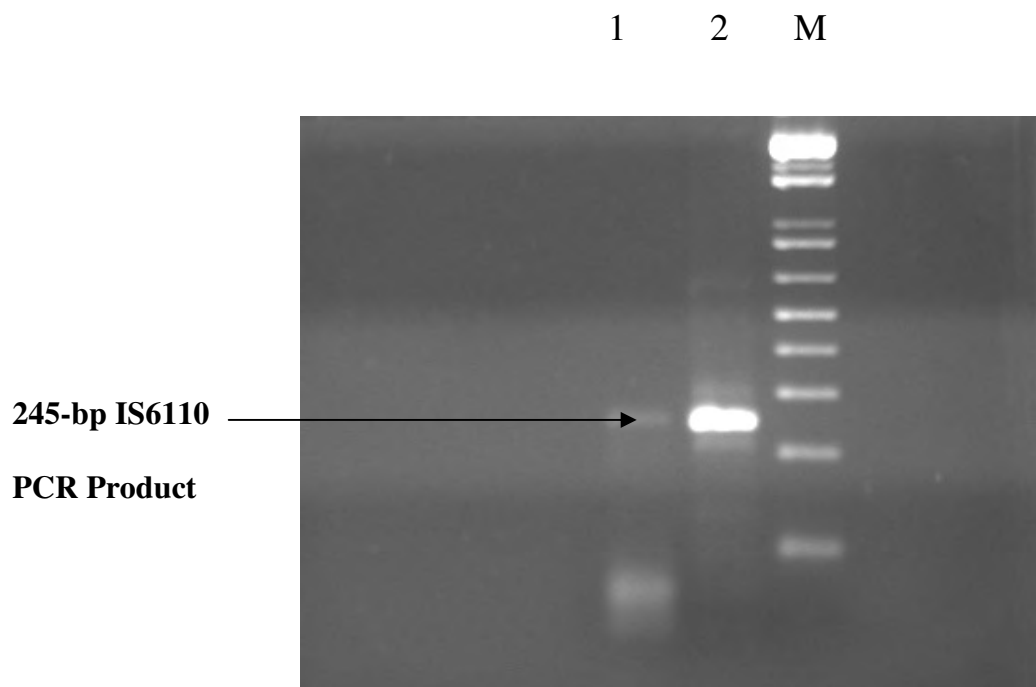


Figure 6 A 245bp IS6110 PCR product from *M. tuberculosis* strain Mt 14323. Lane 1 and 2 are the 245 bp PCR product, lane M is a 1Kb molecular marker.

Due to various problems that include inability to optimize the RFLP analysis, we could not succeed in getting good RFLP pattern for our isolates. Repeatedly we got dark back ground and faint bands on the autoradiograph. However, as it is shown in schematic representation of figure 7, a relatively better autoradiograph was obtained for 10 isolates.

A total of eight distinct RFLP patterns were obtained from these ten isolates. As shown in Figure 5, Lane 8 and 9 had isolates with 3 IS6110 copies that are predominant in Ethiopia.

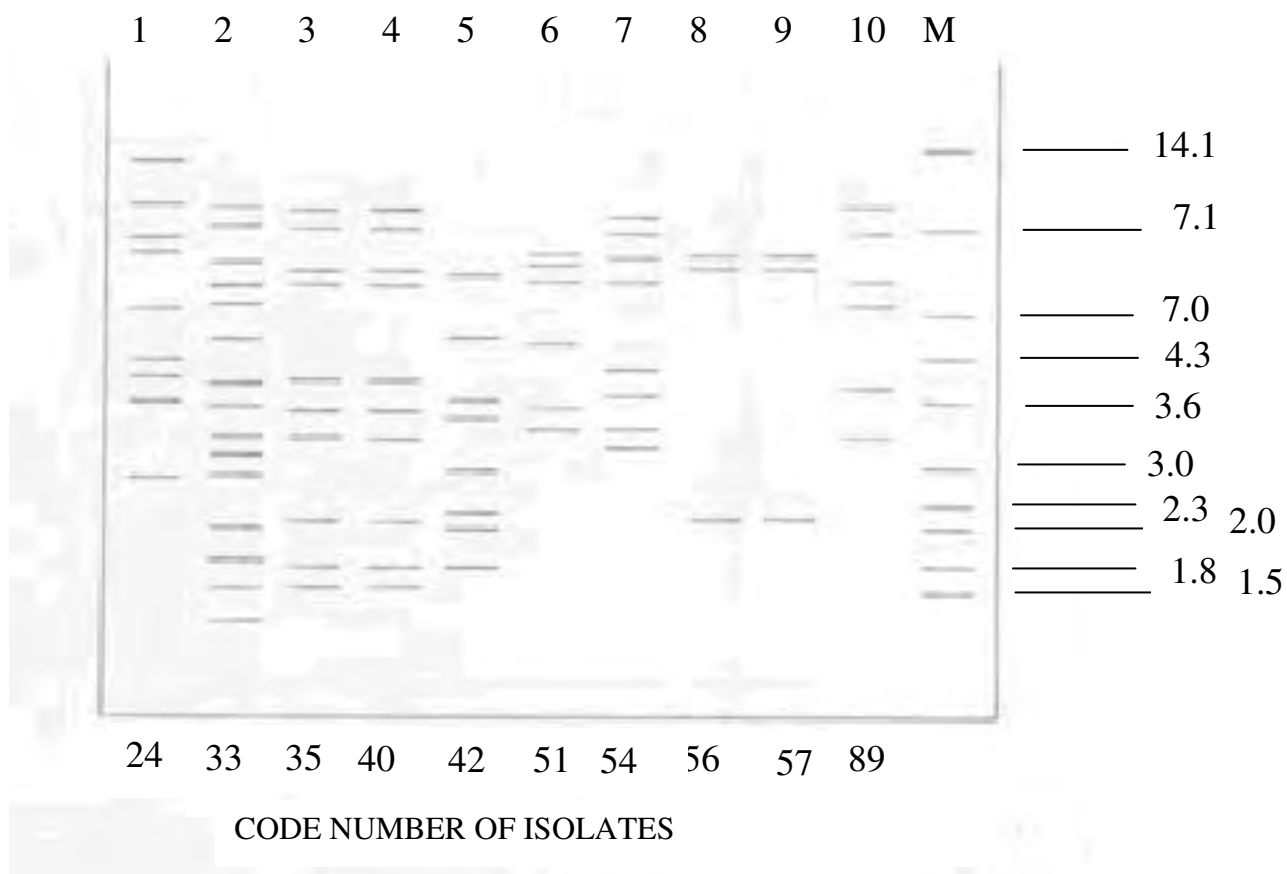


Figure 7 Schematic representation of the RFLP pattern of *M. tuberculosis* strains isolated from 10 patients. Lane M is the reference strain Mt 14323. Numbers on the right indicate sizes of the reference marker in kilobase pairs.

4. Discussion

The demographic profile of the study population shows that a high number of re-treatment cases are males and more number is observed at a specified age group. From the total number of 100 re-treatment cases enrolled for this study 70% were males and about 86% belonged to the age group ranging between 15 and 44. Tuberculosis affects the most productive age group in the population. The reasons for such occurrence are quite variable. One study in Ethiopia showed a high rate of non-compliance to be a characteristic well associated with males (Demissie and Kebede, 1994), but that could be due to the exposure risk one faces because of his differential, social, economic and cultural role in the society. This is not unique to Ethiopia. In most developing countries two third of the reported TB patients are men (WHO, 1998). Comparable results have been reported from Ghana (Hudelson, 1996).

Knowledge of the level of drug resistance is a good indicator of the extent of transmission of the disease and success of national tuberculosis control programme. The level of drug resistance provides an insight into the extent of transmission of drug resistant bacilli in a community as well as the success of the national tuberculosis programme (Shah *et al.*, 2002). Ethiopia, although it is one of the 22 high burden countries that account for 80% of the world's new TB cases, has little information with regard to drug resistance. It is one of the countries that has not been surveyed by WHO/ IUATLD global project on anti tuberculosis. It is as part of an effort to fill the gap that the present survey was conducted. The resistance observed in our patients could be due to primary or acquired resistance.

The result showed that 58% of the strains were resistant to one or more anti- tuberculosis drugs. This clearly shows that there is a high prevalence of resistance to the first line anti-tuberculosis drugs among the re-treatment cases in Addis Ababa. Our result is comparable with a similar study on re-treatment cases in Addis Ababa that showed 50% of the strain to be resistant to one or more of the first line drugs (Abate *et al.*, 1998). The result of surveys conducted in 48 countries by the Global project indicates prevalence rate of drug resistance ranging from 0 to 94% with a median rate of 26.9% among previously treated patients (WHO, 2000a). A high rate of resistance has been observed in our patients compared with many of the countries surveyed by the Global project.

Resistance to isoniazid was found to be 49.3%. It comes first compared to the other three drugs: rifampicin, streptomycin and ethambutol. This figure is not very different from the results of the previous studies. The present study however, showed an increase in resistance to rifampicin and ethambutol. The high rate of rifampicin resistance could be associated with a number of factors: extensive use of the drug for TB and other diseases, malabsorption, non-compliance and single drug administration. It is possible patients with HIV infection may have altered absorption for rifampicin that might lead to the development of rifampicin monoresistance (Peloquin *et al.*, 1996). Though the HIV status of our patients was not known, a high rate of HIV-TB co-infection (42% in adults within the age range of 15 to 49yrs) (WHO, 2003) in Ethiopia indicates that malabsorption could be one reason of rifampicin monoresistance and this could be a recent phenomenon.

A study that aimed to investigate the determinants of drug resistant tuberculosis in 11 countries, has shown that resistance to rifampicin and ehambutol, anti TB drugs recently

introduced in TB programmes were observed in all age groups, suggesting recent infection with drug resistant strains (Espinal *et al.*, 2001).

MDR TB was observed in 29.3% of our patients. This, compared with the prevalence of MDR TB in re-treatment cases, 3.5% (Pattyn *et al.*, 1979) and 12% (Abate *et al.*, 1998) is distinctly high. Based on the second global project the prevalence of MDR TB among previously treated patients ranged from 0 to 48.2% with 9.3% median prevalence. The percentage of MDR cases in previously treated patients of many African countries ranges from 3.3% to 23.1%. Our result shows that a high proportion of patients with MDR cases are living in Ethiopia. Chronic cases and failures are more likely to have MDR strains compared to the other type of patients. Most of the MDR patients were from Addis Ababa, however there were few patients from different regions of the country indicating that MDR is emerging as a problem in various parts of Ethiopia.

Comparison of drug resistance between two groups of patients treated with loose and 3FDC regimen revealed that a statistically significant association has been observed between prior treatment with 3FDC regimen and rifampicin and isoniazid resistance. In St Peter's specialized TB hospital a relatively high level of relapse is observed in tuberculosis patients who had been treated with 3FDC regimen than loose drugs (Dr. Solomon Goshu, Personal Communication, 2002).

Studies carried out in various parts of the world have reported different results regarding the efficacy of 3FDC therapy. A 24 months of follow up survey indicated that patients treated with 3FDC regimen had a higher relapse rate than those treated with loose drugs

(Teo, 1999). On the other hand encouraging results have been reported on the efficacy of 3FDC regimen from various parts of the world. Relatively low prevalence rates of MDR TB have been recorded in Brazil and South Africa where good quality FDCs are claimed to have been used (Pablos- Mendez *et al.*, 1998). One study that had an objective of investigating the efficacy of FDCs revealed the absence of a statistically significant difference in drug resistance among patients treated with FDC and loose drugs (Su and Perng, 2002).

One of the reasons for the occasional failure of FDC in the treatment of TB is because of poor bioavailability (the amount of drug which gets absorbed into the blood) of rifampicin. It appears that the bioavailability of rifampicin in FDC's is easily compromised if strict manufacturing procedures are not followed or poor quality raw materials are used. If the bioavailability of rifampicin is inadequate, treatment failure and emergence of drug resistant tuberculosis could follow (Blomberg *et al.*, 2001). This, when seen in light with the result of a study that has reported 10% (4/40) of all anti- tuberculosis drug samples and 13% (4/30) of the rifampicin samples, obtained in national programs and local and hospital pharmacies from six countries in three continents to have substandard content (Laserson *et al.*, 2001) makes the problem vivid.

Our result indicates a high rifampicin and isoniazid resistance rate in patients treated with 3FDC. However, because of the comparatively small numbers of patients studied, the results from this study may only be used as good indicators of the need for a more systematic study addressing the relative efficacy of FDC. It is also important to note that

such a study is currently being planned with different institutes in Ethiopia and with the support of TDR/WHO.

Rapid detection of drug resistance is essential for adequate treatment and optimal control of tuberculosis. However, most developing countries have limited capacity to perform drug susceptibility testing of tubercle bacilli even for patients failing to respond to the available drugs. Except for some cross sectional studies which were conducted in some major towns, a nationwide drug resistance survey has never been conducted in Ethiopia (Abate, 2002). This is because the available techniques are expensive and labor intensive. Therefore it was one goal of this work to establish and evaluate a simple drug susceptibility assay that can be applied in developing countries like Ethiopia.

MTT assay was previously standardized and evaluated for indirect detection of rifampicin resistance (Mshana *et al.*, 1998; Abate *et al.*, 1998). Our results of the MTT assay were confirmed by the standard proportion method on 7H10 media using strains isolated on LJ media from the same samples. The assay identified 23/75 (30.7%) isolates as rifampicin resistant and 52/75 (69.3%) as rifampicin susceptible. The result matches 97.3% with drug susceptibility result of the proportion method. The mean RODU value of resistant strains was 1.08 ± 0.5 and it was above 0.5. The mean RODU value of susceptible strains was 0.04 ± 0.06 and it was below 0.2. This result was in match with the RODU values for resistant and susceptible strains in standardized indirect MTT assay (Abate *et al.*, 1998; Foongladda *et al.*, 2002; Caviedes *et al.*, 2002).

Only one sputum specimen was collected from each patient instead of three sputum specimens. This reduces the cost of materials, reagents and minimizes inconvenience to the patient.

A total of four tubes (three controls and one rifampicin containing tube) were used for the MTT assay. Therefore our result showed that it is possible to do direct MTT assay using four tubes.

From the total isolates 19.2% gave interpretable result at week one, 67.9% at week two and 100% at week three. Therefore direct MTT assay could give a result with in three weeks of time. The result can be visually read and thus MTT assay could be done in places where spectrophotometer is not available.

A total of eight distinct RFLP patterns were obtained from the ten isolates. Two isolates have identical RFLP profile with 3 IS6110 copies. This three-banded pattern is predominant in Ethiopia (Hermans *et al.*, 1995). Though we could not succeed in getting good RFLP pattern for our isolates, the importance of the method for typing *M. tuberculosis* should not be neglected. RFLP is particularly useful to study recent transmission of tuberculosis during TB outbreak (van Soolingen, 2001).

Conclusion and Recommendation

Control of tuberculosis is threatened by widespread emergence of drug resistance in *Mycobacterium tuberculosis*. This is due to the fact that TB treatment and control is complicated when *M. tuberculosis* is resistant to the action of anti- tuberculosis drugs. Resistance to rifampicin and isoniazid (MDR), in particular, decreases the effectiveness and greatly increases the cost of TB treatment and mortality rate.

An accurate estimation of drug resistance would serve as an indicator of the transmission of drug resistant organisms as well as the efficacy of the national tuberculosis control programme. Moreover, early detection of drug resistance could lead to appropriate control measures to limit further spread of the disease.

In Ethiopia there have been some studies on drug resistance tuberculosis that were mainly focused on primary drug resistance. These studies showed a low prevalence rate of primary drug resistance and multidrug resistance tuberculosis. However, a high rate of drug resistance and MDR was observed in isolates collected from retreatment cases. Therefore, currently, drug resistant and MDR strains appear to be largely originating from patients who are receiving re-treatment.

The present finding suggested that drug resistant *M. tuberculosis* infection are becoming important problem and due attention should be given i.e. recognize that it is more serious problem in the future. Therefore factors related to the outcome must be examined.

The study investigated the prevalence of drug resistance among re-treatment cases in St Peter's TB specialized hospital. Drug resistance profile of the patients who had been treated

with 3FDC and loose drugs were also compared. Furthermore the rapid and simple direct MTT assay to detect rifampicin resistant *M. tuberculosis* was evaluated using our clinical isolates.

The result showed that a high level of drug resistance (58.7%) to one or more anti TB drugs and MDR of 29.3% were in the study population. This figure is high compared to the prevalence rate of drug resistant tuberculosis in different parts of the world. Management of re-treatment TB patients, particularly MDR cases would be better if handled by a special team that conducts operational research and monitors the situation. An upward trend of resistance was observed in the number of isolates resistant to rifampicin, isoniazid and ethambutol.

A statistically significant prevalence of rifampicin and isoniazid resistance was observed more frequently in patients who had been treated with 3FDC regimen than those TB patients treated with loose drugs.

The result of direct MTT assay was obtained within three weeks of time. It was also observed that the result could be interpreted with the naked eye. This makes the method simple and rapid. Current diagnostic strategies should be therefore upgraded to include methods like the direct MTT assay after its validation on a larger scale.

Strain typing using RFLP techniques should be optimized here in our settings, which would have various applications.

Despite an increase in the treatment success rate for tuberculosis in Ethiopia (from 72% of patients registered in 1997 to 81% of patients registered in 2001) (WHO, 2003), an upward trend of re-treatment cases that are harboring drug resistant TB isolates is observed. This problem poses a question into investigating and studying particularly the follow up programme of treatments in DOT that might be the cause of an increase in the rate of re-treatment cases.

This preliminary result requires further study on the efficacy and relapse rate of the 3FDC regimen currently given in Ethiopia. In view of this problem a protocol is being prepared to test the rifampicin bioavailability of 3FDC regimen.

Further study is needed to standardize the direct MTT assay for all drugs particularly for isoniazid, which will help us in diagnosing MDR TB with in three weeks of time.

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Appendix 1

Clinical information from re-treatment cases

I. General

Age (yrs)____ Sex (M/ F) Address _____ Card No.____ Date _____
Patient type (D/F/R/C)

II. Remission period in months (free from symptoms after the last treatment)

_____.

III. Clinical presentation at present

<u>Manifestation</u> (Mark × if present)	<u>Duration (weeks)</u>
Cough ()	_____
Sputum production ()	_____
Haemoptysis ()	_____
Fever ()	_____
Night sweat ()	_____
Chest pain ()	_____
Others (), specify	_____

IV. Treatment previously taken

Anti-TB drugs previously used (Mark × if used, and specify duration in the intensive and continuation phases of treatment)

H () _____ S () _____
R () _____ E () _____

Treatment regimen previously used

Intensive phase

Loose _____
2FDC (specify) _____
3FDC (specify) _____

Continuation phase

Appendix 2

Drug sensitivity test result of *Mycobacterium tuberculosis* isolates

CODE	Drug Sensitivity Test			
	R	H	S	E
1	S	R	R	S
2	S	S	S	S
3	R	R	I	R
4	S	R	S	S
5	S	S	S	S
6	S	S	S	S
7	S	S	S	S
8	S	R	S	S
9	S	I	S	S
10	R	R	S	R
11	S	S	S	S
12	S	S	S	S
13	R	R	R	S
16	S	R	I	S
17	S	S	S	S
18	R	R	R	I
19	R	R	R	I
20	S	R	I	S
21	R	R	R	I
22	S	S	R	S
23	S	S	S	S
24	R	R	R	R
25	S	S	S	S
26	R	R	R	I
27	R	R	I	S
29	S	I	I	I
30	S	S	S	S

CODE	Drug Sensitivity Test			
	R	H	S	E
31	S	S	S	S
33	S	S	S	S
35	S	S	S	S
36	R	R	S	I
37	R	R	R	R
38	R	R	R	S
40	S	S	S	S
41	S	R	S	S
42	S	S	S	S
44	R	R	S	S
45	S	R	S	I
46	S	S	S	S
47	S	I	S	I
48	R	S	S	I
51	S	S	S	I
52	S	S	S	S
53	R	S	S	I
54	R	S	S	I
55	S	S	S	S
56	S	S	S	R
57	S	S	I	I
58	R	R	I	S
59	S	S	S	S
60	R	I	S	S
61	R	R	R	R
62	R	R	I	S
63	S	S	S	S
64	S	S	S	S
65	S	S	S	S
66	S	R	I	I

CODE	Drug Sensitivity Test			
	R	H	S	E
67	S	S	S	S
68	S	S	S	S
70	S	S	S	S
71	R	R	R	I
72	R	R	R	I
80	S	S	S	S
81	S	S	S	S
83	R	R	R	R
85	S	S	S	S
86	S	R	S	I
88	S	S	S	S
89	S	S	S	S
91	R	R	S	I
93	S	R	S	S
94	R	I	R	I
98	S	S	S	S
99	S	R	R	S
100	S	R	S	S

R =Resistant

S = Susceptible

I = Intermediate