ADDIS ABABA UNIVERSITY
SCHOOL OF GRAGUATE STUDIES

PRIMARY DRUG RESISTANCE PATTERNS OF *Mycobacterium tuberculosis* ISOLATES AMONG NEW PULMONARY TUBERCULOSIS PATIENTS IN BAHIR DAR, ETHIOPIA

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By

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TABLE OF CONTENTS

TITLE PAGE

ACKNOWLEDGEMENTS i

LIST OF FIGURES v

LIST OF TABLES vi

ACRONYMS vii

ABSRACT viii

1.INTRODUCTION 1

2.LITERATURE REVIEW 6

2.1.The Genus *Mycobacterium* 6
2.2.The Major TB organism: *Mycobacterium tuberculosis* *7*

2.2.1. Transmission and Pathogenesis 7

2.2.2.Drug resistance in *Mycobacterium tuberculosis* 11

2.3.Global burden of tuberculosis 15

2.4.The Global HIV/AIDS epidemic and Tuberculosis 18

2.5.Tuberculosis in Ethiopia 19

2.6.Laboratory diagnosis of tuberculosis 20
2.6.1.Smear Microscopy 20
2.6.2.Culture 21
2.6.3. Molecular methods 22

2.7.Detection methods of drug resistance in *M.tuberculosis* 23
2.7.1.Phenotypic drug susceptibility testing 23
2.7.2.Molecular methods (Genotypic tests) 25

2.8.Chemotherapeutics against tuberculosis 25

TABLE OF CONTENTS

3.MATERIALS AND METHODS 29

3.1. Study Area 29

3.2.Study population 29

3.3.Specimen collection, transport and processing 30

3.4.Smear examination 32

3.5.Culture 32

3.6.Identification of *Mycobacterium tuberculosi*s species 33
3.6.1.Determination of cord-formation (pattern) 33
3.6.2.Biochemcial tests 33
3.6.2.1.Combined niacin- nitrate reduction tests. 33
3.6.2.2.TCH resistance test 36

3.7.Drug susceptibility testing 36
3.7.1. Antibiotics 36
3.7.2. Media 38
3.7.3. Inocula 38
3.7.4.Susceptibility test 39
3.7.5.Control strains 39
3.7.6.Interpretation of susceptibility test results 39

3.8. Statistical Analysis 39

4.RESULTS 40
4.1. Classification of patients and *Mycobacterial* isolates 40

4.2. Identification of *M. tuberculosis* species 42

4.2.1.Biochemical characterization/tests 43

4.3. Patterns of primary resistance 44

5. DISCUSSION 49

5.1.Limitations of the study 54

6.CONCLUSIONS AND RECOMMENDATIONS 58

REFERENCES 61

ANNEXES

I. Sputum specimen collectionform.....77

II. A *Mycobacterium* growth chart.....……..78

III. Drug susceptibility test result recording form..... .79

LIST OF FIGURES **PAGE**

1.The development and spread of drug- and multi-drug resistant tuberculosis.....14

2. Distribution of primary drug resistance by age and sex. 48

LIST OF TABLES **PAGE**

1.Geographical settings with prevalence of MDR-TB greater than 3% among new cases.....17

2. Age and sex distribution of PTB patients included in the study in Bahir Dar, Ethiopia…….. 43

3. Summary of patterns of primary resistance of PTB patients.....45

4. Primary resistance with age and sex distribution of new PTB patients 49

ACRONYMS USED (IN THE TEXT)

AFB	Acid-Fast Bacteria
AIDS	Acquired Immunodeficiency Syndrome
Anti-TB	Anti-tuberculosis
BCG	Bacillus Calmette Guerin
ATCC	American Type Culture Collection
DOTS	Directly Observed Treatment, Short Course
EPTB	Extra Pulmonary Tuberculosis
HIV	Human Immunodeficiency Virus
IUATLD	International Union Against Tuberculosis and Lung Disease
L	Liter
L-J	LÖwenstein-Jensen
µg	Microgram
MIC	Minimum Inhibitory concentration
µM	Micrometer
ml	Milliliter
MDR-TB	Multi Drug Resistant Tuberculosis
NTLCP	National Tuberculosis and Leprosy Control Programme
PTB	Pulmonary tuberculosis
SLS	Sodium Lauryl Sulfate
TB	Tuberculosis
TCH	Thiophene-2-carboxylic acid hydrazide
WHO	World Health Organization
Z-N	Zeihl-Neelsen

ABSTRACT

The increase in drug resistant tuberculosis, and particularly multi-drug resistant tuberculosis (MDR-TB) as well as the neglect of discovery and development of new anti-TB drugs that are active against TB/ MDR-TB at present, increased the need for knowledge of the prevalence and patterns of primary resistance among *Mycobacterium tuberculosis* isolates in the community. A total of 76 *M. tuberculosis* isolates were recovered from sputa specimens of newly diagnosed pulmonary tuberculosis (PTB) patients in Bahir Dar, Ethiopia and confirmed by culture and standard biochemical tests on L-J media. The specimens were collected between February and April 2001. Drug susceptibility testing was performed according to the indirect proportion method. The prevalence and patterns of primary resistance were determined and any association between patients' characteristics (sex and age) and primary resistance were analyzed using chi-square test. The results of the present study showed that primary mono-resistance was highest to streptomycin (14.5 %) followed by isoniazid (2.6 %). All isolates were susceptible to rifampicin and ethambutol. Primary resistance to any drug was found in 14 of new PTB patients (18.4 %); and any primary resistance to streptomycin was 15.8 %; any isoniazid resistance was 3.9 %; any rifampicin resistance was 1.3 %, and it was nil to ethambutol. The rate of Primary MDR-TB was 1.3 %. Single drug resistance was higher in males (n=8, 61.5 % of mono-resistant cases) than in females (n=5, 38.5 %). Primary resistance to a single drug was found in 4 patients in the age group 15-24 (30.8 %), 4 in the group 25-34 (30.8 %) and 5 in the group 35-44 (38.5 %). Thus, the proportion of patients that had primary mono-resistance was similar in all age groups and the age groups between 15-44 were totally affected by primary drug resistant strains. Primary MDR-TB (with HRS pattern) occurred in one patient (1.3 %) belonged to the age group 25-34. Single drug resistance to streptomycin was found in 8 males (73 %) and in 3 female patients (27 %). Isoniazid mono-resistance was found only in female patients (n = 2, 100 %). The age range of TB patients that had primary resistant isolates of *M. tuberculosis* were between 15-44 years, with mean age of 27.4 years. However, primary resistance was not associated with age and gender ($P>0.05$). The drugs isoniazid, ethambutol and rifampicin remain drugs of choice to treat the majority of drug susceptible TB cases in Bahir Dar. The results of this study underscore the need for periodic monitoring of drug resistance and improving in TB control performance both at local and country level.

1. INTRODUCTION

Mycobacterium tuberculosis is the major causative agent of tuberculosis (TB), a chronic infectious disease that remains an important public health problem throughout the world (WHO, 2000). Robert Koch first described the tubercle bacillus in 1882 and demonstrated that *Mycobacterium tuberculosis* was the aetiological agent of tuberculosis (Falkow, 1988; Daniel *et al.,* 1994). After Koch's progress, in the early 1920s, Calmette and Guerin isolated an attenuated culture of *M. bovis* and demonstrated its function as a vaccine (BCG vaccine) against TB (Bloom and Murry, 1992). Finally, efficient anti-tuberculosis drugs were discovered at the very beginning of the anti-biotherapy era (WHO, 1998b). As a result, for many years TB has been both a preventable and a curable disease.

However, in spite of these early advances, TB causes more adult deaths worldwide than any other infectious disease. About a third of the world's population harbors *M. tuberculosis* and is at risk for developing the disease (Daffe and Etienne, 1999; Raviglione *et al.,* 1999). Each year, there are an estimated 8 million new cases of TB and 3 million deaths from the disease (Dye *et al*., 1999; Freidag *et al*., 2000). Indeed, at current rates, it has been estimated that up to one half of a billion people will suffer from TB in the next 50 years. Thus, the infection will prove fatal to more than 200,000,000 of them (Kochi, 1994; Pelicic *et al.,* 1998).

Despite the fact that efforts made to control TB started in the early 1960s, TB still remains an important public health challenge in Ethiopia. Some hospital data show that TB is among the leading causes of morbidity and mortality in the country (NTLCP, 1997a). Netto *et al*., (1999) reported that the country is among the top 22 TB burden countries of the world. Likewise, a

World Health Organization (WHO) report indicates that Ethiopia is among one of a high TB incidence countries in Africa (WHO, 2000).

Several factors including worsening social conditions (Hudelson, 1996; Kazionny *et al*., 2001) and low vaccine efficacy of the BCG against the most common pulmonary TB (Fine, 1995), explain the discrepancy between the early successes in containing TB and the present alarming situation. Moreover, recent years have seen increased incidence of TB in both developing and industrialized countries (Cohn *et al*., 1997). The widespread emergence of drug-resistant strains of *M. tuberculosis* and HIV/TB co-infection are the main reasons to such TB increase (WHO/IUATLD, 1997; Di Perri *et al*., 1989; Selwyne *et al*., 1989)

Drug resistant tuberculosis, and particularly multi-drug resistant TB (MDR-TB, defined as cases of TB due to strains of *M. tuberculosis* resistant to at least isoniazid and rifampicin) is an increasing health problem in the world (Zwolska *et al*., 2000). Recent Global Anti-tuberculosis Drug Resistance Surveillance studies have shown that drug resistance was ubiquitous. Furthermore, MDR-TB strains have also been described worldwide (WHO, 2000). Globally, about 50 million people are already infected with drug resistant strains of *M. tuberculosis* and acquired resistance is more common than primary resistance (Becerra *et al*., 2000). A prevalence of primary MDR-TB as high as 14.4 % was reported in Latvia and acquired MDR-TB rate as high as 54.4 % and 89 % was reported in Latvia and in Azerbaijan, respectively (Schluger, 2000).

Of all patterns of drug resistance, multi-drug resistant tuberculosis is the one that attracted international attention. This is due to the reduced response of multi-drug resistant (MDR) strains to the most effective and cheaper short-course chemotherapy with first-line anti-

tuberculosis drugs (WHO/IUATLD, 1997). As a consequence, this would lead to higher mortality and treatment failure rates and increased periods of disease transmission within a community (Gonzalez *et al*., 1999). Not only are patients infected with MDR-TB strains less likely to be cured, but also treatment with second line anti-tuberculosis drugs has been associated with considerable cost to the society and most of these drugs are highly toxic (Bastian and Colebunders, 1999). For instance, the death rate for MDR-TB patients treated with second line anti-TB drugs is 40-60 %, the same as untreated individuals who develop disease due to drug susceptible strains. For people co-infected with HIV/MDR-TB, this rate is as high as 80 % (Goble *et al*., 1993; Park *et al*., 1996). In addition, failure of new drug discovery or development for TB/drug-resistant TB compounds the problem.

Nevertheless, the possibility of β-lactam or other existing anti-tuberculosis drugs is being investigated for their potential use against multi-drug resistant tuberculosis (Ramaswamy and Musser, 1998). In general, poor TB control, HIV/AIDS epidemic, low socio-economic status, substance abuse, war, ethnic conflicts and migration contribute to the development and spread of MDR-TB, which, however, is manageable with proper health control measures (Chaisson *et al*., 1999; Kazionny *et al*., 2001).

It is evident that drug resistance, particularly multi-drug resistance is a major threat to tuberculosis control programme. It increases the specter of untreatable disease. Besides, its detection requires routine drug susceptibility testing of isolates from each patient, which is costly. In most resource poor countries of the world, detection and management of drug resistant TB is very expensive. Thus, the appearance and spread of MDR-TB in these countries would lead to higher case fatality rate (Kochi *et al*., 1993). Earlier, the link between drug resistance and tuberculosis control programme has been noted. So, the importance to

monitor drug resistance patterns in improving performance of tuberculosis control and in reducing the disease burden has also been advocated recently (Canetti *et al*., 1969; Kim and Hong, 1992;WHO/IUATLD, 1998). Therefore, in resource poor countries of the world with high TB burden, drug susceptibility testing is helpful as a drug resistance surveillance tool (WHO/IUATLD, 1997).

In Ethiopia, some hospital-based studies suggest that the problem of drug resistant TB, both for single or multiple drugs, exists (Abate, 1999). The results of these studies showed that primary resistance rate to any drug was as high as 32.5 % (Mitike *et al*., 1997), and it was 1 % for primary MDR-TB (Demissie *et al*., 1997). In addition, acquired MDR-TB rate as high as 12 % was reported in Addis Ababa (Abate *et al*., 1998). Moreover, the nature of the problem varies from place to place. On the other hand, based on all available information, no national data indicating the magnitude of the problem have been established in Ethiopia yet. Therefore, it is essential to obtain information on patterns of drug resistance in different settings of the country.

The study area, Bahir Dar is the capital of Amhara region, northwest Ethiopia, 565 Km away from Addis Ababa. Between the years 1999 and 2000,TB was the major cause of morbidity seen among patients who visited two governmental health institutions found in Bahir Dar (BHC, 2000; FHH, 2000.Unpublishedreport). Without doubt, there is no published information on drug resistance patterns of anti-tuberculosis drugs in the study area. This study, therefore, was designed to examine new pulmonary tuberculosis cases for harboring drug resistant strains of *M.tuberculosis.* If these strains could be found, the study would also determine the extent to which they were transmitted within the community. It was also

intended to contribute to knowledge of drug-resistance by providing preliminary information about the situation of primary drug-resistance patterns in the study area.

The most important benefit from this study would be to call attention to what is a critical and neglected aspect in TB control of our country, as it (i.e. primary drug resistance) would demonstrate the transmission of drug resistant strains of *M. tuberculosis.* Thus, such data might be used to avoid treatment of the corresponding disease with inappropriate anti-tuberculosis drugs. Also, the information obtained from the study would be useful to make recommendations on the performance of TB control programme.

2. LITERATURE REVIEW

2.1.The Genus *Mycobacterium*

Mycobacteria are aerobic, non-motile, acid-fast, rod shaped bacteria. They belong to the class Actinomycetes; order Actinomycetales and family Mycobacteriaceae (Broke, 1978; Wayne and Kubica, 1986; Shinnick and Good, 1994). The genus *Mycobacterium* constitutes obligate parasites, saprophytes, and opportunistic commensals that can be isolated from clinical samples, soil, water or other environmental sources (Kubica, 1984).

All members of the genus *Mycobacterium* are further classified into different species based on colony characteristics, growth rate, nutritional requirements, biochemical tests and genomic studies (Kubica and Good, 1981; Rogall *et al*., 1990; van Embden *et al*., 1993; Doolittle, 1999). The genus *Mycobacterium* consists of 83 species and of these, about 30 species are noted to be pathogenic to human beings either as opportunistic or strict pathogens (Abate, 1999). For instance, *M. tuberculosis* complex comprises of *M. tuberculosis, M. africanum, M. bovis* and *M. microti,* which are slow growing obligate pathogens causing TB in humans (the former two), bovines, and avians, respectively. Even though *M. bovis* and *M. africanum* are known to cause TB in man, they contribute to a small fraction of the disease (IUATLD, 1998). In addition, the complex lacks inter-strain genetic diversity, and nucleotide changes are very rare (Strauss and Falkow, 1997; Magdalena *et al.,* 1998).

2.2. The Major TB Organism: *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is an aerobic, non-motile, rod shaped, acid-alcohol fast bacterium with a guanine-cytosine (G+C) content of 65.6 % (range 61-71%). Its optimum growth temperature is between 35 ^{0}C and 37 ^{0}C. When grown on solid medium like Lowenstein-Jensen or Middlebrook 7H10 agar, strains of *M. tuberculosis* produce non-pigmented visible colonies between 3 and 8 weeks (Broke, 1978; Kubica, 1984; Wayne and Kubica, 1986). It is the major intracellular human pathogen that causes tuberculosis and differs radically from other groups of the genus *Mycobacterium* in that several enzymes are involved in its lipid metabolism (Cole, *et al*., 1998).

2.2.1. Transmission and Pathogenesis

Mycobacterium tuberculosis is transmitted from person to person mainly through air-borne droplet nuclei (i.e. bacilli-bearing particles less than 100μm in diameter) released from the lungs via the nose and mouth when a patient with active TB coughs, sneezes or speaks. Droplet nuclei can remain suspended in small air currents ready to be inhaled, until removed by ventilation. Infection begins when the bacilli reach the alveoli and phagocytic alveolar macrophages are the first cells infected by *M.tuberculosis* (Bloom and Murry, 1992). Some of the bacilli may be killed immediately; others may multiply within the macrophages. Infrequently, but especially in immune-suppressed persons and in children, the bacilli spread to other sites in the body. This dissemination sometimes results in life-threatening meningitis.

One of the key steps in the pathogenesis of *M. tuberculosis* infection is the ability of the tubercle bacillus to enter and replicate within the macrophages of its human host (Des Jardin and Schlesinger, 2000). It is well established that single gene encoded virulence factors are important for the pathogenesis of *M. tuberculosis* (Pelicic *et al.,* 1998). These include: catalase peroxidase, katG gene encoded enzyme, which protects the bacillus against reactive oxygen species such as hydroxyl radical, superoxide and hydrogen peroxide produced by the phagocyte; macrophage colonizing factor (possibly encoding invasin), encoded by mce gene, which is responsible for the ability of the bacillus to bind and invade macrophages; a sigma factor gene (sigA), which is involved in the transcription specificity of RNA polymerase or to promote the transcription of a subset of genes involved in virulence (Cole *et al*., 1998); and isocitrate lyase (ICL), icl gene encoded enzyme that promotes persistence of the bacillus within inflammatory macrophages (McKinney *et al.,* 2000).

Furthermore, bacillary enzymes like phospholipases C, lipases, and esterases, which might attack cellular, or vacuolar membranes, and several proteases could act as virulence factors. For instance, phospholipases act as contact dependent haemolysin. These proteins may well be secreted or surface exposed to play a role in invasion of host cells (Cole *et al.*, 1998).

In addition, the mycobacterial cell wall is also essential in pathogenesis by allowing intracellular survival of the bacillus and it also renders the bacillus impermeable to common drugs (Brennan and Draper, 1994; Kolattukudy *et al*., 1997). The mycobacterial cell wall components such as mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan may contribute to mycobacterial longevity, trigger inflammatory host reaction and act in pathogenesis (Cole *et al*., 1998).

Recently, it has been demonstrated that the bacteria may persist intracellularly not only in macrophages but also in non-professional phagocytic cells (Hernandez-Pando *et al*., 2000). These include fibroblasts, endothelial- and epithelial-cells in lung tissue in the absence of tuberculous lesions. This is actuality in spite of the dominant view that latent organisms exist in old classic tuberculous lesions (Rook and Bloom, 1994; Hernandez-Pando *et al*., 2000).

Usually, within 2 to 8 weeks after initial infection, most individuals are able to mount an effective immune response. In people within intact immune systems, infected phagocytic alveolar macrophages present pieces of the bacilli, displayed on their cell surfaces, to immune lymphocyte called the T-cell. When stimulated, T-cells release an elaborate array of chemical signals. Some of the T-cell signals produce inflammatory reactions; other signals recruit macrophage reactivation and pro-inflammatory cytokine production (Enarson and Rouillon, 1998). As a result, they kill *M. tuberculosis* and wall-off infected macrophages in tinny, hard

grayish nodules (tubercles) and thereby prevents dissemination of bacilli (Parish *et al.*, 1998). Hence, the host's immune response limits the proliferation of the bacilli and produces a long lasting partial immunity, both to new infection and to the reactivation of old infection (Sutherland *et al.*, 1976). However, in primary TB infection, even when successfully controlled by the immune system, not all bacteria are eliminated. Some remain dormant but viable (persistent) bacilli (Bloom and Murry, 1992).

If, however, as acquired immunity wanes through aging or immune suppression, progression or breakdown of infection leads to active disease in about 10 % of infected cases. Active disease (TB) usually results from the spread of the bacilli from the alveoli through the blood stream or lymphatic system to other sites of the body, usually in the lungs or local lymph nodes. Notably, the clinical manifestations of active TB depend on the site of *M. tuberculosis* spread and accordingly, TB can be grouped into two major forms: pulmonary form (PTB)-TB developed in the lungs and extra-pulmonary form (EPTB)-TB developed outside the lungs. Pulmonary tuberculosis (PTB) is the most common type of TB comprising about 80-85 % of all TB cases, while EPTB is found in about 15-20 % of cases (WHO, 1996a). In general, *Mycobacterium tuberculosis* accounts for 98% cases of PTB spread via air-borne droplet nuclei and 70% of EPTB form (Selwyne *et al*., 1989).

Although many bacilli are killed, a large proportion of infiltrating phagocytes and local tissue e.g. lung parenchyma cells, die as well producing characteristic solid caseous (cheese-like) necrosis/granuloma in which bacilli may survive but not flourish. If the necrotic reaction expands, breaking in to a bronchus, a cavity is produced in the lungs allowing a large numbers of bacilli to spread with coughing to the outside. As the disease progresses, the granulomas may liquefy, perhaps a result of released hydrolases from inflammatory cells, which creates a

rich medium for the proliferation and spread of the bacilli. The pathologic and inflammatory processes produce the characteristic weakness, fever, chest pain, and, when a blood vessel is eroded, bloody sputum (Bloom and Murry, 1992; Vynnycky and Fine, 2000).

2.2.2. Drug Resistance in *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is naturally resistant to many antibiotics, making treatment difficult. This resistance is due mainly to the highly hydrophobic cell envelope acting as a permeability barrier. In addition, many potential resistance determinants are also encoded in the genome. These include drug-modifying enzymes such as β-lactamases and amino glycoside acetyltransferases, and many potential drug efflux systems responsible for extruding the drugs (Neu, 1992; Brennan and Draper, 1994).

Recently, the DNA sequence of *M. tuberculosis* was found extraordinarily well conserved, and mutations in its genome are almost exclusively associated with drug resistance (Musser, 1995). During replication, drug resistance in *M.tuberculosis* develops by sequential acquisition of mutations in target genes with a frequency that has been defined (Riska *et al.,* 2000; Walsh, 2000) but varies from drug to drug (Mitchison and Nunn, 1986; Grange, 1990). For instance, mutations resulting to rifampicin resistance occur at a rate of 10^{-10} to 10^{-7} per cell division, leading to an estimated resistance prevalence of 1 in 10^{8} bacilli. Mutations resulting to isoniazid resistance occur at a rate of 10^{-9} per cell division, leading to an estimated resistance prevalence of 1 in 10^{6} bacilli. Hence, resistance to rifampicin develops less frequently than isoniazid. Earlier, it was demonstrated that bacilli greater than 10^{7} are common in lung cavities in TB infected patients (Canetti, 1965). Thus, resistant mutants evolve in the absence of anti-microbial exposure, but they are diluted within the majority of drug susceptible bacilli.

Usually, mutations resulting in resistance to the various different classes of drugs are genetically unlinked. Therefore, spontaneously occurring MDR is virtually impossible, since there is no single gene involving in such process. For instance, the probability of developing resistance to both isoniazid and rifampicin is 1 in 10^{14} (10^{6} x 10^{8}, for isoniazid and rifampicin respectively). Which is highly unlikely, given that cavity lesions usually contain between 10^{8} and 10^{9} bacilli (Isman and Madson, 1989). This is in fact, one of the essential reasons for the use of multi-drug regimens in the treatment of TB (Cohn *et al.,* 1959; WHO, 1996a).

If one of those mutations confers resistance to an applied antibiotic i.e. exposure to a single drug due to irregular supply, poor quality, inappropriate prescription and /or poor adherence to treatment, where all sensitive bacilli are killed, the resistant one grow, fill the space vacated by its dead neighbors and become the dominant variant in the population. If an antibiotic is at sub-therapeutic levels such as in a combination of drugs where the minimum inhibitory concentration (MIC) of one drug may be optimal, out growth of resistant bacilli is practically guaranteed (Mitchison, 1950). This phenomenon is called acquired resistance. For this reason, TB must be treated with optimal multi-drug therapy (Schluger *et al.,* 1996). Moreover, subsequent transmission of such bacilli to other persons may lead to disease, which is drug resistant from the outset, a phenomenon known as primary resistance (Figure 1).

According to Canetti, (1965), every drug active against *M. tuberculosis* is bound to select for resistance. So, the presence of anti-microbial provides the selective pressure, which favors a resistant cell, which then multiplies to become predominant. This is, especially important in patients with a large load of bacilli, such as those with extensive cavitations disease. Thus, resistance of *M. tuberculosis* to anti-mycobacterial drugs is a man-made amplification of

spontaneous mutations in the genes of the tubercle bacilli. Likewise, multi-drug resistant isolates can arise. This is because random mutations in genes that encode target genes for the individual anti-TB agents are selected by sub-therapeutic drug doses (Mitchison, 1998). Sub-therapeutic drug levels can occur due to processes like treatment errors and poor-adherence to treatment protocols (Mahmoudi and Isman, 1993; Pablos-Mendez *et al.,* 1996).

The emergence of drug resistant strains of *M. tuberculosis* in a population has been associated with a variety of management, health provider and patient related factors (Addington, 1979; Fox, 1983; Barnes, 1987; Sumartajo, 1993; Pablos-Mendez *et al*., 1997). Finally, a crucial element in the emergence of drug resistance is the lack of a properly organized system to ensure prompt diagnosis and effective treatment (Reichman, 1997). For this reason, the level of anti-TB drug resistance in a population is an indicator of the effectiveness of a National TB Programme (WHO/IUATLD, 1998; Coninx *et al*., 1999).

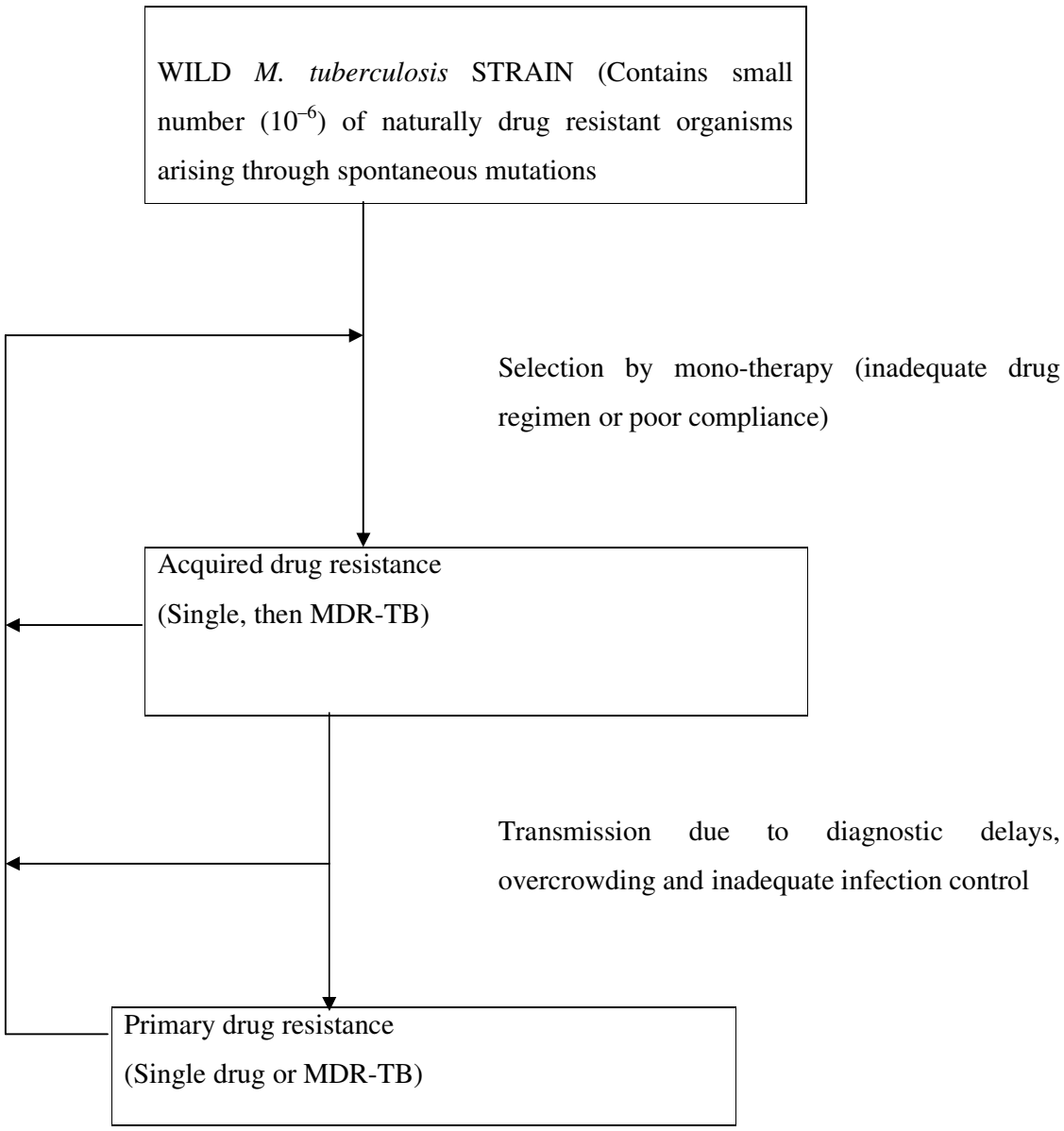

Figure 1. The development and spread of drug- and multi-drug resistant tuberculosis

2.3. Global Burden of Tuberculosis

Despite the availability of effective short course chemotherapy, millions of people have died from tuberculosis. In 1993, the gravity of the situation led the World Health Organization (WHO) to declare tuberculosis a global emergency (Kochi, 1994; Raviglione *et al*., 1995). However, TB is undoubtedly increasing in incidence and prevalence in most countries.

About a third of the world's population is estimated to be at risk of infection with *M. tuberculosis* (Daffe and Etienne, 1999), leading to 8 million new cases a year (Boshoff and Mizrahi, 2000). Notably, there are 17.3 million existing cases of TB throughout the world. Indeed, 80 % of all incident TB cases of the world are in 22 countries with more than half the cases occurring in five South East Asian countries namely, Bangladesh, China, India, Indonesia, and Pakistan (Raviglione *et al*., 1999). Also, nine of the ten countries with the highest incidence rates found in Africa are in sub-Saharan Africa with estimated incidence rate of 259 per 100,000 persons, as compared with Europe, the lowest incidence rate, 51 per 100, 000 persons (Netto *et al*., 1999).

In addition, mortality in adults due to *M. tuberculosis* accounts for 26 % of all preventable adult deaths globally (Ramaswamy and Musser, 1998). Unfortunately, 95 % of TB cases and 98 % of TB deaths are in developing countries with 75 % of cases in economically active age group between 15 and 50 years of age (WHO, 1998b).

Furthermore, between the years 1998 and 2030, 225 million new cases and 79 million deaths as a result of *M. tuberculosis* infection are expected (Bastian *et al*., 2000). This increase will be greatest in sub-Saharan Africa, which has the highest rate of growth of the young adult population and the least effective TB control programmes (Dye *et al*., 1998).

To date, drug resistant TB is a global problem and has spread at an alarming rate. However, a more serious aspect of the problem has been the appearance and occurrence of micro-epidemics of TB due to MDR strains of *M. tuberculosis*, which poses a major threat to the individual patient as well as to the community (WHO/IUATLD, 1997). Table 1 presents geographical settings with prevalence of primary MDR-TB greater than 3 %.

Table 1: Geographical Settings with Prevalence of primary MDR-TB (>3%) (WHO/IUATLD, 1997; WHO, 2000).

Setting/Country	Prevalence of MDR/TB (%) between the Years:	
	1994-1997	1996-1999
Argentina	4.6	-
Cote d' Ivoire	5.3	-
Dominican Republic	6.6	-
Estonia	10.2	14.1
Henan province (China)	-	10.8
India (Delhi state)	13.3	-
Islamic Republic of Iran	-	5.0
Ivanovo oblast (Russia)	4.0	9.0
Latvia	14.4	9.0
Mozambique	-	3.5
Tamil Nadu state (India)	-	3.4
Thailand	3.8	-
Tomsk oblast (Russia)	-	6.5
Zhejiang province (China)	-	4.4

2.4. The Global HIV/AIDS epidemic and Tuberculosis

Human immunodeficiency virus (HIV) infection is an increasing problem and has become an additional challenge to TB control efforts (WHO, 1997). The World Health Organization (WHO) estimated that 32 % of the 40 million people living with HIV/AIDS worldwide in the year 2000 were also infected with TB. Of these 32 %, it is likely that 30-40% will develop active disease. This number is increasing and alarming since there is a direct association between HIV infection and reactivation of latent TB or progressive TB from newly acquired infection (Selwyne *et al*., 1989; Daley *et al*., 1992).

For people dually infected with HIV/TB, the lifetime risk of developing TB is about 50 % with the yearly risk of 10 %, as compared with the yearly risk of 0.2 % and the life-time risk of 10 % in HIV negative, TB infected individuals (Narian *et al.*, 1992). Likewise, drug/multi-drug resistant TB spreads rapidly as a consequence of inadequate treatment practices amplified by HIV. However, it is noted that only when drug resistance becomes prevalent in the community is HIV a risk factor for acquiring it (Chaisson *et al.*, 1999).

Although some data suggest that certain HIV-positive patients may be at elevated risk of developing rifampicin resistant tuberculosis, perhaps due to drug absorption problems (Ramaswamy and Musser, 1998), evidence from different settings suggests that HIV- infected TB patients are no more likely to develop drug resistance than HIV-negative TB patients (WHO, 2000). Additionally, short course chemotherapy regimens have shown similar early response rates in both HIV-positive and negative TB patients and there is increasing evidence that long term TB relapse rates are similar in both groups. While one study has

suggested that relapse may be more common in HIV sero-positive individuals (Ridzon *et al.,* 1998), no distinction was made in other study between relapse and reinfection (Dean *et al*., 2002).

In several countries in sub-Saharan Africa, 30-70% of TB Patients are HIV positive. Thus, the impact of HIV infection has placed an enormous burden on the general health services and on TB control in Africa, which is devastating. At present, HIV related TB coupled with drug resistant TB is ported enormous difficulties for TB control programmes in many parts of the world (WHO, 1997). Such troubles have been demonstrated even in countries with good functioning TB control Programme (Cantwell and Binkin, 1996; Sansila *et al*., 1998).

2.5.Tuberculosis in Ethiopia

Ethiopia has a high rate of tuberculosis infection and the disease is one of the significant public health problems in the country (NTLCP, 1997a). Yet, no reliable data indicating the national incidence and prevalence figures of TB is established. On the other hand, in Ethiopia the annual incidence of new TB cases is estimated to be 260 per 100,000 people with a prevalence of 367 per 100,000 people (Netto *et al.*, 1999). More recently, WHO reported that Ethiopia is among one of a high TB incidence countries in Africa (WHO, 2000).

To date, the continued explosion of HIV related TB and socioeconomic related factors, such as: poverty, migration, population explosion, war and ethnic conflicts are believed to be the reasons to the alarming resurgence of TB in the country (Abate, 1999). In Ethiopia, the prevalence of TB/HIV co-infection is estimated to be 15.4 %; and 30 % of new TB cases may have HIV infection (Dye *et al*., 1999).

Moreover, the emergence of drug resistant strains of *M. tuberculosis* may compound the problem. A few of hospital-based studies have been revealed that the problem of drug resistant TB both for any drug and multiple drugs exists in the country. Although periodic monitoring of drug resistance patterns seems a neglected task, based on the results of these studies, the level of primary resistance to any anti-tuberculosis drug was as high as 32.5 % and primary multi-drug resistance TB ranged between 0.4-1% (Wolde *et al.*, 1986; Mitike *et al.* 1997). In addition, the prevalence of acquired resistance to one or more anti-TB drugs was in the order of 31.6-56 % and the prevalence of acquired multi-drug resistance TB was as high as12 % (Pattyn *et al*., 1979; Abate *et al*., 1998; Gebeyehu *et al.*, 2001).

2.6.Laboratory Diagnosis (methods) of Tuberculosis

Laboratory diagnosis of TB is helpful to confirm the clinical diagnosis given on the basis of signs and symptoms of the disease with radiographic findings that may show evidence of TB infection in the form of cavities. Both are important to obtain information suggestive of TB (Lumb *et al*., 1999). Due to its importance in control Programme, the laboratory diagnosis of TB will be directed to pulmonary tuberculosis and specifically to sputum (sputum containing) specimens (Kubica, 1984). Available methods for the laboratory diagnosis of TB are stated below.

2.6.1.Smear Microscopy

The observation of acid-fast bacteria (AFB) in a stained smear examined under the microscope is often the first evidence of the presence of mycobacterial disease (Kent and Kubica, 1985). Smear microscopy, although it is a simple, cheap and rapid method, is neither specific nor sensitive enough. However, for management of sputum smear positive PTB cases, this method has been the top priority of the WHO TB Control Programme (Kochi,

1991). On the other hand, before a suspected patient is considered smear negative, at least two sputa samples per patient have to be examined (NTLCP, 1997b; WHO, 1998a).

Acid-fast bacteria smear preparations include the direct and indirect (concentrated) ones that can be examined under the microscope using either Ziehl-Neelsen (Z-N) or fluorescent based staining techniques (Kent and Kubica, 1985). Since Z-N staining technique is slower and less sensitive than the latter, which is expensive, examination of concentrated smear or multiple smears prepared from repeatedly induced samples could increase its sensitivity (Nelson *et al.*, 1998; Al Zahrani *et al*., 2001).

2.6.2.Culture

Culture is usually required for the laboratory confirmation of TB and it enables specific identification of the mycobacterial pathogen. This is accomplished after the tubercle bacilli have been recovered on primary isolation media (Kubica, 1984). The bacilli can be recovered using either solid media such as egg-based (e.g. Lowenstein-Jensen in tubes) and agar-based (e.g. Middlebrook (MB) in tubes or on plates) or liquid media like MB or Dubos broth. Then, subjected to standard biochemical or gene probe tests for specific identification (IUATLD, 1998; WHO/IUATLD, 1998).

For primary isolation of *Mycobacterium,* smear positive early morning sputa samples from each patient should be collected aseptically in hermetically sealed standard containers before commencement of treatment. Prior to culture, the collected specimens can be kept at a temperature of + 4 ^{0}C until a maximum of 2 weeks (Engbaek and Bentzon, 1964a; Engbaek and Bentzon, 1964b; Kestle and Kubica, 1967; Banda *et al*., 2000), in order to retard growth

of contaminants and to preserve mycobacteria, or the specimens can be processed for culture immediately after collection.

Culture method involves:

(1) **Specimen processing**. Sputum samples are digested and decontaminated to liquefy the organic debris surrounding the microbes, so that the decontaminating agents can kill the contaminants and to allow surviving mycobacteria access to nutrients of primary isolation media (Kubica, 1984). The digestion decontamination procedures include: acetyl-cystein alkaline, sodium hydroxide; zephiran trisodiumphosphate; and sodium laurylsulfate methods (Engbaek *et al.,* 1967; Kubica, 1984);

(2) **Inoculation and Incubation.** The processed sediment is then inoculated on primary isolation media and incubated at a temperature of 37 ^{0}C incubator containing 10 % CO_2. Although, *M.tuberculosis* is strict aerobe some authors suggest that 10 % CO_2 stimulates its growth on solid media (Kubica, 1984). Usually positive cultures can be observed between 3 to 8 weeks and all positive cultures are stored until further tests at a temperature of -20 ^{0}C or for sometime at 4^{0}C or even at room temperature (Kubica and Good, 1981; WHO/IUATLD, 1998).

All procedures involving sputum samples containing mycobacteria should be carried out in a biological safety cabinet designed for this purpose and, during specimen transport, the containers should be sealed and packed in order to avoid risk of reduced viability of bacilli, and contamination leading to infection (WHO, 1993); and

(3) **Identification**. All culture positive isolates should be identified using standard biochemical tests (Kent and Kubica, 1985) or standard gene probe tests (WHO/IUATLD, 1998).

In addition to culture, rapid but expensive automated systems like the BACTEC 460 radiometric and the non-radiometric one, Mycobacterial Growth Index Tube (MGIT) have been developed and provide early detection/isolation of mycobacteria in less than three weeks (Roberts *et al*., 1983). Culture, although it offers improved sensitivity, is time consuming and requires high cost safety facilities. Despite these limitations, culture has been recommended as a cost effective method for assessing and monitoring the prevalence of drug resistant TB in any TB control Programme (IUATLD, 1998).

2.6.3.Molecular Methods

These methods are advanced and nucleic acid based techniques, which show promise as more rapid, sensitive and specific for identification of mycobacteria and detection of *M. tuberculosis* directly from processed specimens or indirectly from primary culture, but are rather expensive. Moreover, they are helpful for strain characterization (van Embden *et al.,* 1993; Cohn and O'Brien, 1998). Molecular methods include amplification of species-specific nucleic acid sequences, PCR amplification and restriction enzyme analysis, hybridization with species-specific oligonucleotide probes with or without prior DNA amplification, nucleic acid sequence determination, and restriction fragment length polymorphism (RFLP) analysis (van Embden *et al*., 1993; Kurepina *et al*., 1998; Magdalena *et al*., 1998).

2.7. Detection methods of drug resistance in *M. tuberculosis*

2.7.1.Phenotypic drug susceptibility testing

This method is essential to measure the ability of *M. tuberculosis* proliferation in a drug containing solid or liquid media. It can be performed either directly on clinical specimens confirmed AFB smear positive or indirectly on a culture isolate of *M. tuberculosi*s (Canetti *et*

al., 1969; Heifets, 1991). The following four standard methods are available for indirect drug susceptibility testing:

- The Resistance Ratio (RR) method**.** A ratio is obtained by dividing the minimum inhibitory concentration (MIC) of the test strain with the MIC of standard susceptible strain. RR of 8 defines drug resistance, while RR of 2-drug susceptibility. RR between 2 and 8 is considered intermediate and, on repeated test, it can be considered as resistant (Canetti *et al*., 1969).
- The Proportion Method. This method consists in calculating the proportion of resistant bacilli present in a strain. Resistance is defined as more growth on drug containing media as compared to the growth on a drug free control containing ten fold serially diluted tubercle bacilli suspension or a ratio obtained (i.e. above the critical proportion) by dividing the number of colonies grown on drug containing medium with the number of colonies grown on drug free control. The critical proportion of resistant bacilli required to define a strain as resistant is 1% for all drugs tested (Canetti *et al*., 1969; Kubica, 1984; Heifets, 1991).
- The Absolute Concentration Method. Drug resistance is defined as the growth of 20 colonies on media containing a defined concentration of the test drug (Canetti *et al*., 1969).
- The BACTEC460 Radiometric Method**.** It is a variant of the proportion method. The growth of mycobacteria in drug free control and drug containing vials is quantified as growth index based on the amount of ^{14}C-labeled CO_2 produced. A strain is resistant if the difference in growth index (GI) of control is less than that of the vial with drugs (Roberts *et al*, 1983).

2.7.2.Molecular methods (Genotypic tests)

These methods are rapid and can be used both on culture-based and directly on clinical specimens (e.g. sputum) harboring *M. tuberculosis* (Rinder *et al.,* 2001). Detection of specific mutations in target resistant genes is the basis for genotypic tests.

Molecular methods include direct sequencing of PCR products; those based on altered gel migration patterns: Single stranded conformational polymorphism (SSCP) RRFLP analysis, RNA/RNA duplex, base-pair mismatch assay, hetro-duplex analysis, and dideoxy fingerprinting; and those based on altered hybridization to probes: such as rRNA/DNA bioluminescence labeled probe assay, a reverse hybridization based line probe assay, and DNA chip hybridization (Telenti *et al*., 1993; Cohn and O'Brien, 1998; Riska *et al.*, 2000).

2.8. Chemotherapeutics against Tuberculosis

Anti-tuberculosis drugs are natural or synthetic/semi-synthetic products used to treat active TB. They act on *M. tuberculosis* through their (1) bactericidal activity i.e. early bactericidal activity (ability to kill the actively multiplying tubercle bacilli) thereby, break chains of transmission in the community or through their sterilizing activity (the ability to kill semi-dormant bacilli which are resistant to early bactericidal activity of other drugs imposing the risk of reactivation/ relapse, and (2) through their bacteriostatic activity i.e. the ability to nullify growth (Jindani *et al*., 1980; Winder, 1982).

In general, three proven drug targets are known to exist: (1) bacterial cell wall biosynthesis, (2) protein synthesis, and (3) nucleic acid biosynthesis. The mechanism of action of the anti-mycobacterial agents involves inhibition or inactivation of specific cellular sites that play key

roles in the synthesis pathway or transcription. In the treatment of active TB, drugs with sterilizing effect play a role in shortening the duration of chemotherapy by virtue of their ability to kill semi-dormant bacilli within macrophages thereby avoiding the risk of relapse. This is, in fact, the basis of short course chemotherapy and multi-drug therapy, using a combination of anti-TB drugs that possess high sterilizing and early bactericidal activity (Burman, 1997). Moreover, the use of combination therapy with optimal therapeutic dose prevents the emergence of resistance. However, the use of combinations of distinct antibiotic classes that work on different drug targets concurrently is of therapeutic importance than using two components work together to neutralize a single target. At present, short course chemotherapy of TB uses, a combination of first-line anti-tuberculosis drugs to treat drug susceptible strains of *M. tuberculosis* and a combination of second-line alternative medicines to treat TB caused by drug/multi-drug-resistant strains (Mitchison, 1985; Isman, 1993; Bastian and Colebunders, 1999).

First line anti-tuberculosis drugs. These are front-line drug of choices used against wild type strains of *M. tuberculosis*. They are cheap, less toxic, and effective drugs that are and have been used worldwide, and are the basis for the current directly observed therapy, short course (DOTS), a strategy for care of TB patients (i.e. 6-8 months rifampicin based TB therapy). Front-line chemotherapeutic agents against *M. tuberculosis* (TB) include:

1. Isoniazid. It is a synthetic analog of nicotinic acid with a molecular weight of 137, and is water-soluble. It is a selective bactericidal agent against *M. tuberculosis* complex and has been used since 1952 (WHO, 1997). Isoniazid is a pro-drug requiring activation by catalase peroxidase enzyme (encoded by katG gene), to its biologically active form, isonicotinic acid, in order to act on its gene targets/site, leading to inhibition of mycolic acid biosynthesis (Cole *et al*., 1998).

2. Rifampicin. It is a semi-synthetic derivative of a natural antibiotic Rifamycins B produced mainly by *Amycolatopsis mediterranei* (Lal *et al*., 1995). Rifampicin has a molecular weight of 822.9, and is soluble in chloroform, ethyl acetate, methanol, dimethyl sulfoxide or water (at low pH). The drug has been used since 1966 for the treatment of TB and recently used to treat leprosy. It is highly bactericidal with a potent sterilizing effect against tubercle bacilli and its mechanism of action is that it binds to the β-subunit of DNA dependent RNA polymerase (encoded by rpoB gene) resulting in inhibition of transcription and RNA elongation (Levin and Hatful, 1993; Telenti *et al*., 1993).

3. Streptomycin. It is an aminoglycoside antibiotic derived from *Streptomyces griseous,* with a molecular weight of 581.6, and is water-soluble. Streptomycin is a moderately potent bactericidal drug against tubercle bacilli in a neutral or alkaline environment, and it has been used to treat TB since 1944. It acts by impairing protein synthesis at the bacterial ribosome. It binds to 16S rRNA (encoded by rrs gene) and inhibits translation initiation and detrimentally affects translation fidelity (Sreevastan *et al*., 1996; Riska *et al.,* 2000).

4.Ethambutol. It is a potent bacteriostatic synthetic drug that has been used against TB since 1965, has a molecular weight of 277.2, and is soluble in water. The drug is active against all strains of *M. tuberculosis, M. bovis* and other mycobacteria. Ethambutol acts to inhibit the synthesis of the arabinogalactan component of the mycolic acid/cell wall, via embCAB gene cluster i.e. genes encoding highly similar proteins that are presumed to function as arabinosytransferase, an enzyme involved in polymerization of arabinogalactan from arabinose (Wolucka *et al*., 1994).

5.Pyrazinamide. It is a structural analog (derivative) of nicotinamide with a molecular weight of 131.1 and in water. It has been used against TB since 1952. This drug is a pro-drug which is converted to pyrazinoic acid via pyrazinamidase (i.e. an enzyme encoded by pncA gene) of the tubercle bacilli under acidic condition that plays a key role in shortening the duration of

chemotherapy by virtue of its ability to kill semi-dormant populations of *M. tuberculosis* (Heifets, 1991;WHO, 1997). The mycobacterial target of the activated form remains elusive (Scorpio and Zhang, 1996; Boshoff and Mizrahi, 2000).

Second-line anti-tuberculosis drugs. Drugs such as: ethionamide, cycloserine, thioacetazone, kanamycin, capreomycin, viomycin, amikacin and fluoroquinolones are used as secondary or alternative agents to treat TB caused by strains of *M. tuberculosis* resistant to the commonly used primary anti-tuberculosis drugs (WHO, 1996b). These drugs are commonly used in combination with some of the first-line drugs to which the strain is susceptible (Ramaswamy and Musser, 1998). While second-line drugs are recommended as an important components of DOTS-plus strategy (i.e. directly observed MDR-TB therapy using second line anti-TB drugs on the basis of individual patients drug susceptibility test result), except thioacetazone, all are expensive and some have multiple side effects e.g. toxicity (WHO/IUATLD, 1997; Isman, 1998).

3.MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Bahir Dar, a capital of Amhara Region, located in the northwestern part of Ethiopia, and 565kms away from Addis Ababa. In 1999, the population of Bahir Dar town, estimated by the Region's Bureau of Planning and Economic Development, was 125,025 (BoPED, 1999). Two governmental health institutions (one hospital and one health center) are found in the town. In addition to other medical care, both serve TB patients who visit them for diagnosis and treatment of TB under DOTS TB Control Programmee. Between September 1999 and August 2000, TB was among the major reported causes of morbidity frequently seen in both health institutions. In this a one-year period, a total of 400 smear-positive new TB cases were registered for treatment (BHC, 2000; FHH, 2000). Unpublished report.

3.2.Study population

The study population consisted of all smear positive PTB patients with no history of prior treatment against TB. These TB patients who visited both health institutions between February and April 2001,were simultaneously included in the study. Information on history of previous treatment, age, sex and residence area of each TB patient was recorded in both institutions using a format (annex I). A case of new smear positive PTB was defined as a TB patient who denied previous treatment against TB up on direct questioning by the physician, confirmed smear positive to at least two consecutive early morning on-spot sputum smears by microscopy and with clinical as well as radiographic findings suggestive of TB.

3.3.Specimen collection, transport and processing

Specimens were collected from patients with new smear positive TB who were eligible for the study. They were asked to submit a single, an early morning sputum specimen each using a hermetically sealed sterile standard container before commencing treatments. A total of 76 specimens were collected and kept in a refrigerator at a temperature of +4 ^{0}C, until they were

transported. Cold boxes with dry ice (in bags) were used to transport all the collected sputa samples to TB laboratory in Armauer Hansen Research Institute (AHRI), Addis Ababa (Ethiopia). The specimens were kept in cold room (+4 ^{0}C) immediately at arrival, and then were digested, decontaminated and further homogenized for culturing according to the Sodium laurylsulfate method (Kubica, 1984).

The Sodium Lauryl Sulfate (SLS) Digestion-Decontamination procedures used were:

1. To one part (1 ml) of sputum in a centrifuge tube 3 parts (3ml) of 3 % sodium lauryl sulfate solution (i.e. a solution prepared by dissolving 30 gram of sodium lauryl sulfate in 1000 ml of sterile deionized water heated to 60 ^{0}C and by adding 10 g ram of NaOH) was added.
2. The tubes (with sputum and SLS solution) were mixed by putting them on a shaker for 20 minutes and centrifuged at 3000 x g (relative centrifugal force) for 15 minutes
3. The supernatant was removed and the sediment was neutralized with 0.45 % sulfuric acid bromocresol purple solution (i.e. a solution of 0.45 ml concentrated H_2SO_4, 100 ml deionized water, and 2 ml bromocresol purple) by adding two drops at a time until the color changes from violet to yellow (strong purple).

Then, the neutralized sputum suspensions were mixed very well and transferred into three Lowenstein-Jensen media (3 drops per a medium using 1 ml disposable plastic pipette), and incubated at 37 ^{0}C in a 10 % CO_2-90 % air incubator. In addition, sputum specimens were processed for smear microscopy using 5 % sodium hypochlorite (NaOCl; household bleach) concentration method and, after a day, all smears were heat-fixed.

Hypochlorite methods used for concentration of *Mycobacterium* were:

1. To one ml sputum in a centrifuge tube an equal volume (1ml) of 5 % sodium hypochlorite solution (i.e. NaOCl; household bleach) was added.
2. After Stopper securely, contents of the tube were mixed to liquefy.

3. The tube was let to stand for 15 minutes at room temperature.
4. The mixture was diluted with sterile water (8 ml) and centrifuged at 3000 x g for 15 minutes.
5. The supernatant fluid was decanted.
6. A smear was prepared by transferring a drop of the sediment (using 1-ml capacity disposable plastic tube) on to a slide.
7. The smear was allowed to air dry for a day.

Finally, the smear was heat-fixed by passing it 3-4 times through the blue cone of a burner flame in biological safety cabinet.

All containers that hold specimens were carefully sealed and packaged to avoid leakage or breakage that may lead to infection and contamination in transit. Furthermore, all processes of sputum specimens, culture and subsequent tests were handled in the biological safety cabinet in the TB laboratory (WHO, 1993).

3.4.Smear Examination

All heat-fixed sputum smears were examined for the presence of AFB using Z-N staining technique, and a 100 x oil immersion objective microscopy confirmed the presence of AFB.

Ziehl-Neelsen staining Technique

1. The heat-fixed slide was flooded with carbolfuchsine solution (i.e. a solution of 10 ml of 3 % fuchsine solution and 90 ml of 5 % phenol solution) and heated gently for 2-3 minutes to steaming with a Bunsen flame.
2. It was left for 3-5 minutes to cool, and then washed in running tap water.
3. It was decolorized in 25 % H_2SO_4 for 10-15 minutes, and washed in running tap water.
4. Next, it was dehydrated in 96 % ethyl alcohol for 10-15 minutes, followed by washing the slide in running tap water.
5. Then, the slide was counter stained with methylene blue solution (i.e. a solution prepared by dissolving 0.3 gram of methylene blue chloride in 100 ml distilled water) for 1-2 minutes, followed by washing the slide in running tap water.
6. Finally, the slide was air-dried and examined under oil immersion, 100x objective. Bacilli were appeared red against blue background.

3.5.Culture

Each processed specimen (i.e. processed for culture by sodium laurylsulfate method) with an amount of 0.1 ml was inoculated into three Lowenstein-Jensen slopes. The tubes were incubated at 37 ^{0}C in a concentration of 10 % CO_2 incubator and examined for the appearance of visible colonies at weekly intervals up to 8 weeks. Growth was recorded on a chart (annex II). Finally, all culture positive isolates were stored in triplicate at a temperature of –20 ^{0}C until further test i.e. from each culture positive isolate a loopful of colonies were transferred

into nunc tubes that contained 1ml of a 1:1 mixture of 50 % glycerol solution and Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, Michigan, USA).

3.6.Identification of *Mycobacterium tuberculosi*s species

3.6.1.Determination of cord-formation (pattern)

Cord-formation of *Mycobacterium* was detected by stained smears prepared from the culture growth using the Z-N staining technique. A loopful of colonies was transferred on a glass slide, heat-fixed, stained and examined under 100x oil immersion objective microscope. Typical serpentine-like cord pattern/long filament and acid-fast bacilli suggest the presence of mycobacteria (Law, 1960; Heifets and Good, 1994).

3.6.2.Biochemcial tests

Identification of *M. tuberculosis* species was performed using standard biochemical tests: the combined niacin-nitrate reduction (i.e. niacin production and nitrate reduction) and thiophene-2-carboxylicacid hydrazide (TCH) resistance tests (IUATLD, 1998).

3.6.2.1.Combined Niacin- Nitrate reduction tests

M. tuberculosis accumulates the largest amount of niacin and it is a strong nitrate reducer (Kubica *et al*., 1975).

-Niacin production test

- Chemical reagents used for the test were: ethanol, 95 %, aniline, 4 %, and cyanogen bromide, 10 %. Ethanol (95 %) was prepared by mixing 95ml of absolute ethanol (98 %) with 5 ml sterile distilled water, and it was used to prepare aniline, 4% (4ml aniline in 96 ml of 95 % ethanol). Cyanogen bromide (10 %) was prepared by dissolving of cyanogen bromide, 5 gram in 50ml of sterile distilled water. Aniline, 4

% and cyanogen bromide, 10 % were prepared using a brown bottle and stored at a temperature of 5 ^{0}C.

- Control strain. *M.tuberculosis* (ATCC 35836) and *M.gastri/ M.avium* complex were used as positive and negative test organisms control, respectively. In addition, a Lowenstein-Jensen tube of un-inoculated medium was used as a negative reagent control.

-Nitrate Reduction Test

- Chemical reagents used were: $NaNO_3$, 0.01M in phosphate buffer, 0.02M (pH 7.0), prepared by dissolving the following in 100 ml of sterile distilled water: $NaNO_3$, 0.085gram, KH_2PO_4, 0.117gram and $Na_2HPO_4.12H_2O$, 0.485 gram sterilized by autoclaving at 121^{0}C for 15 minutes, a 1:2 dilution of concentrated HCl (i.e. 50 ml of concentrated HCl was added in 50 ml of sterile distilled water containing reagent bottle); 0.2% aqueous solution of sulfanilamide (0.2 ml of sulfanilamide was added in 98.8 ml of sterile distilled water); aqueous solution of N-naphthyl ethylene amine dihydrochloride (0.1 ml) i.e.0.1 ml of it was added in 99.9 ml sterile distilled water; and zinc powder. The last two aqueous solutions were stored in brown bottles at 5 ^{0}C.

- Control strain. *M. tuberculosis* (ATCC 35836) and *M. bovis* (Vaccine strain Bacillus Calmette Guerin (BCG) 390955 B) were used as positive and negative test control, respectively. In addition, an L-J tube of un-inoculated medium was used as a negative reagent control.
- Test organisms. All culture positive a 4 weeks old subcultures (single isolate from each patient) grown on Lowenstein-Jensen media were tested for identification.

Combined Niacin- Nitrate reduction test procedure

1. Buffered nitrate substrate solution (i.e. a solution of 0.01 M $NaNO_3$ in 0.02 M, phosphate buffer, pH 7.0), 2 ml was added to the test culture (i.e. a 4 week old culture on L-J) and the tube was placed up right in a 37 ^{0}C water bath for 2 hours. Pipette tip was used to dislodge some colonies into the substrate.
2. After the 2- hour incubation, 0.6 ml of the substrate solution was removed to a clean sterile test tube for the niacin test.

2.1.a. For the nitrate reduction test, its reagents were added directly to the substrate solution in the remaining culture slant tube i.e. tubes from the water bath were removed and acidified with 1 drop of a 1:2 dilution of concentrated HCl.

2.1.b. Next, 2 drops of a 0.2 % aqueous solution of sulfanilamide and 2 drops of a 0.1% aqueous solution of Naphthylethylene diamine dihydrochloride were added in order.

2.1 c. Immediate formation of pink to red color was considered as a positive result.

2.1.d. If no color change, a small amount of zinc dust was added; this catalyzes immediate reduction of nitrate to nitrite with formation of a red color (confirmation of a true negative reaction). If there was no color change following the addition of powdered zinc, the organisms may have reduced nitrate beyond nitrite; then the test was repeated to confirm this reaction (if confirmed, this would represent a positive nitrate reduction).

2.1.e. A reagent control without organisms (negative) and known positive (*M. tuberculosis*) and negative (*M. bovis*) control organisms were included throughout the test.

2.2.a. For the niacin test, aniline solution, 0.5 ml of 4 % and cyanogen bromide, 0.5 ml of 10 % were added into a test tube containing 0.6ml of the nitrate substrate solution (extract) removed from the water bath earlier.

2.2.b. The solution was observed for an immediate yellow color formation (positive result)

2.2.c appropriate positive (*M. tuberculosis*) and negative (*M.gastri/M.avium* complex) control cultures as well as a tube of un-inoculated medium as a reagent control were included throughout the test.

3.6.2.2.Thiophene-2-Carboxylicacid Hydrazide (TCH) Resistance test

TCH resistance test is used to distinguish between *M. bovis* (susceptible) and *M. tuberculosis* (resistant) strains (Kent and Kubica, 1985).

The drug TCH (Sigma Chemicals Co., Germany) at a critical concentration of 2 µg/ml was tested against all culture positive isolates of mycobacteria (as explained in this text on PP. 36-41).

3.7.Drug susceptibility testing

3.7.1. Antibiotics

Antibiotics ethambutol (Sigma chemicals Co., Germany), isoniazid (Sigma chemicals Co., Germany), rifampicin (Sigma chemicals Co., Germany) streptomycin (Sigma chemicals Co., Germany) and TCH (Sigma chemicals Co., Germany), each were used to prepare stock

solution(s) required for preparing drug containing media. A stock solution of each drug was prepared in a proper solvent at concentrations higher than the maximum final concentrations needed in 100 ml of test medium i.e. Middlebrook 7H10 solid medium (Inderlid and Saltfinger, 1995).

Preparation of stock solutions

Isoniazid. Isoniazid, 20 mg was dissolved in 20 ml phosphate buffer saline (PBS), pH 7.2, to prepare stock solution1. Stock solution 1 of isoniazid was then diluted 1 in 5 (i.e. 2ml of stock 1 was added in to 8ml PBS) to get stock solution 2 of isoniazid.

Streptomycin. Streptomycin, 200 mg was dissolved in 20 ml phosphate buffer saline (PBS), pH 7.2, to prepare its stock solution 1.This solution was further diluted 1 in 5 (i.e. by transferring 2ml of stock 1 in to 8ml PBS) to get its stock 2.

Ethambutol. Ethambutol, 200 mg was dissolved in 20 ml phosphate buffer saline (PBS), pH 7.2, to prepare its stock solution1. It was further diluted 1in2 to get stock solution 2 (i.e. 5 ml of stock 1 was added in to 5 ml PBS).

Rifampicin. A stock solution of rifampicin was prepared by dissolving 20 mg of the drug in 2 ml concentrated ethanol (95%), and followed by dissolving in 18 ml PBS.

Thiophene-2-Carboxylic acid hydrazide (**TCH).** A stock solution of TCH was prepared by dissolving 20 mg of the drug in 10 ml PBS, pH 7.2.

Each stock solution was aseptically filter sterilized using 0.2 μM filter membrane, in a biological safety cabinet. Finally, small aliquots of each stock solution was distributed into nunc-tubes and stored in a deep freezer at a temperature of – 70 ^{0}C.

3.7.2. Media

Middlebrook (MB) 7H10agar (Difco Laboratories, Detroit, Michigan, USA), supplemented with OADC, 10 % i.e. oleic acid- Albumin Dextrose Catalase enrichment (Becton Dickinson, Sparks, MD, USA) and glycerol, 0.5 % was used to prepare susceptibility test media according to the manufacturer's recommendations. The media required for the test were drug free control and drug containing ones, and prepared as follows: MB 7H10 agar medium, 19.5 gram was mixed with 950 ml of sterile distilled water and 5 ml of glycerol, heat dissolved, and distributed in a 100 ml amount in to sterile bottles. Then it was sterilized by autoclaving at 121 ^{0}C for 15 minutes, cooled at a temperature of 50 ^{0}C in a water bath and aseptically 10 ml of OADC enrichment was added to each bottle containing 100 ml of the autoclaved medium. A 0.1 ml of each stock drug aliquots was added to the corresponding medium to give the required final concentrations of 1 and 0.2 µg of isoniazid per ml, 10 and 2 µg of streptomycin per ml, 10 and 5 µg of ethambutol per ml, stock 1 and 2 respectively, and 1µg of rifampicin per ml as well as 2 µg of TCH per ml, from their corresponding stocks. Simultaneously, some of the enriched media with no drug were used to prepare drug free medium. Since, both media had a problem of solidification, immediately after the addition of OADC, they were aseptically distributed in a 4 ml amount in to a sterile test tubes. Finally, all media were dried in a slanted position for 30 minutes and stored at a temperature of +4 ^{0}C in dark. To maintain the potency of each drug, the drug solutions were added in to the corresponding medium after sterilization of the medium.

3.7.3. Inoculum preparation

For the preparation of inoculum, a 3 to 4 week old culture of *M. tuberculosis* strains grown on L-J slants at 37 ^{0}C under 10 % CO_2 pressure was used. A loopful of representative culture

grown on L-J was transferred into a sterile screw capped test tubes with 6-8 sterile glass beads, diluted by adding 3-4 drops of PBS, pH 7.2, and the bacilli were homogenized by vortexing for 5 minutes. Then 3ml of PBS was added, and let to stand for 15 minutes to get a bacillary suspension. Each suspension was adjusted to McFarland No. 1 standard solution (i.e. a solution prepared by adding 0.1 ml of 1 % $BaCl_2$ to 9.9 ml of 1 % H_2SO_4), to yield isolated countable colonies on at least one of the dilutions, upon serial dilutions and subsequent inoculation. Next, each bacterial suspension was serially diluted ten fold up to 1: 1000 (i.e. by serially transferring 1ml suspension into 9 ml sterile distilled water). Finally, the 1in 10 and 1in1000 dilutions were inoculated for the indirect drug susceptibility test.

3.7.4.Susceptibility test

Susceptibility tests were performed using the indirect standard proportion method (Inderlid and Saltfinger, 1995). A 0.1 ml (4 drops using a 1 ml capacity disposable plastic tube) of bacterial suspension diluted ten fold (1:10) was inoculated in to media containing different concentrations of drugs and a drug free medium. In addition, a 0.1 ml of bacterial suspension diluted 100 fold (1:1000) was inoculated into a single drug free control medium per each test. Then all inoculated slants were incubated at 37 ^{0}C in 10 % CO_2 incubator for 21 days. They were checked daily for contamination. Finally, on the 21st day the results were recorded on a chart (annex III)

3.7.5. Control strains

Standard streptomycin-resistant *M. tuberculosis* H_{37}Ra (ATCC 35836) and rifampicin-resistant *M. tuberculosis* H_{37}Rv (ATCC 35838) strains were used as a reference strain per test (i.e. for every 10 isolates tested) and for every batch of media prepared. A vaccine strain *M. bovis* BCG 390955 B (Copenhagen, Denmark) was used as TCH susceptible reference strain.

3.7.6.Interpretation of susceptibility test results

On the 21st day reading of test results was made by counting the number of colonies grew on each of drug containing and drug-free medium. Simultaneously, colony counts were recorded on a chart. Resistance was expressed as the percentage of colonies that grew on critical concentrations of the drugs tested. The proportion of resistant *M. tuberculosis*/bacilli existing in the strain was calculated by dividing the number of colonies obtained on drug containing medium with the number of colonies on drug free control medium when the number of colonies on drug free control was between 50 and 150 (i.e. for both drug-containing and drug free media inoculated with 1 in 10 diluted bacterial suspension). The critical proportion of resistant bacilli required to define a strain as a resistant was 1% for each of the 4 drugs and TCH tested i.e. above 1%, as resistant; and below 1%, as susceptible (Kubica, 1984). Alternatively, the result was interpreted by comparing the number of colonies on a drug-containing medium (i.e. inoculated with 1 in 10 diluted bacterial suspension) with that of a drug free control inoculated with 1 in 1000 diluted bacterial suspension. If the number of colonies on a drug-containing medium is higher than that of the control, it indicates resistance. The presentation of data was based on mutually exclusive categories of resistance as mono-resistance and combined resistance including multi-drug resistance (WHO/IUATLD, 1998). A table was drawn up to describe the proportion of *M. tuberculosis* strains with mono-resistance to each drug, and to different combination of drugs, among patients that had primary resistant strains (Table 3).

3.8. Statistical Analysis

Differences in primary resistance patterns between patients' characteristics (age and gender) were compared using the chi-square test. A p-value of < 0.05 was accepted as indicating statistical significance.

4. RESULTS

4.1.Classification of Patients and Mycobacterial Isolates

A total of 76 isolates of mycobacteria recovered from an equal number of new smear positive pulmonary tuberculosis patients were included in this study. The growth of mycobacterial isolates was confirmed by acid-fast microscopy. Accordingly, in all culture positive isolates the presence of serpentine-like cord pattern and acid-fastness were observed indicating that all the isolates were mycobacteria.

Of the 76 new smear positive patients (new PTB cases) included in the analysis, 33 patients were residents in Bahir Dar town (43.4 %) and the remaining 43 (56.6 %) were from its surroundings. Regarding gender, 50 of the patients were male (65.8 %) and 26 were female patients (34.2 %). They were aged between 12 and 66 years with mean age of 29.2 years. Most of the patients (76.9 %, 20/26) of females and (78 %, 39/50) of males belonged to the age groups 15-44 years (Table 2).

Table 2: Age and sex distribution of PTB patients included in the study from Bahir Dar, Ethiopia

	12-14	15-24	25-34	35-44	45-54	55-64	≥ 65

Age/sex	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Male (n=50)	5 (10)	19 (38)	10 (20)	10 (20)	3 (6)	2 (4)	1(2)
Female (n=26)	4 (15.4)	5 (19.2)	9 (34.6)	6 (23.1)	2 (7.7)	0	0
Total (n=76)	9	24	19	16	5	2	1

n= number of TB cases

4.2.Identification of *M. tuberculosis* species

4.2.1. Biochemical characterization/test

All the 76 isolates were TCH resistant and gave positive test result for niacin production and nitrate reduction, indicating that all the 76 isolates recovered by culture were *M. tuberculosis* species.

4.3. Patterns of primary resistance

All of the 76 isolates of *M. tuberculosis* tested to high concentrations of streptomycin, isoniazid and ethambutol were susceptible to all these three drugs.

Of the 76 new smear positive pulmonary tuberculosis patients studied, 62 patients (81.6 %) excreted bacilli susceptible to all four drugs, while the remaining 14 new pulmonary tuberculosis patients (18.4 %) had strains of *M. tuberculosis* resistant to one or more drugs i.e. the overall primary resistance obtained by adding together single drug resistance to each drug and other patterns of resistance to more than two drugs. The patterns of primary drug resistance in *M. tuberculosis* isolates are shown in Table 3. Most of the patients (13/14, 92.3 %, or 17.1 % of the total, 13/76) excreted primary mono-resistant strains: single drug resistance to streptomycin was found in eleven cases (14.5 %), which was the highest followed by single drug resistance to isoniazid in two cases (2.6 %). No mono-resistance to rifampicin and ethambutol was observed.

Primary resistance to three drugs (HRS) was identified in one patient (1.3 %) and the patient was infected with multi-drug resistant strain of *M. tuberculosis*. None of the isolates (patients) showed resistance to isoniazid and rifampicin, ethambutol plus other drugs and to all four drugs.

Table 3: Summary of patterns of primary resistance of PTB patients

In Bahir Dar, Ethiopia

	Primary resistance n (%)
Total tested	76 (100)

Fully sensitive	62 (81.6)
Any resistance	14 (18.4)
Mono-resistance	
H	2 (2.6)
R	0 (0)
E	0 (0)
S	11 (14.5)
HR resistance	
HR	0 (0)
HRS	1 (1.3)

H= isoniazid, R= rifampicin, E= ethambutol, S= streptomycin, n=number of PTB cases

Total resistance calculated by adding together the number of cases for each drug from each pattern of resistance was 15.8 % for streptomycin, 3.9 % for isoniazid and 1.3 % for rifampicin, but this was nil to ethambutol (Table 3).

Distribution of primary resistance by sex and age group (years) is shown in Figure 2. Primary mono-resistance was significantly higher in males (n= 8,61.5% of mono-resistant cases) than in females (n=5,8.5% of mono-resistant cases). Mono-resistance was found in four patients in the age group 15-24 (30.8 %) four aged 25-34 (30.8 %) and five aged 35-44 (38.5 %). Thus, the age group between 15 and 44 was affected by monoresistant strains of *M. tuberculosis*.

Primary MDR-TB with HRS pattern occurred in one (1.3 %) of the 76 patients, and he belonged to the age group 25-34. Regarding each gender by age group, any primary resistance was found in three males in the age group 15-24, and the same number of male cases had primary mono-resistance to streptomycin. In this age group one female patient had isoniazid mono-resistant strain of *M. tuberculosis*.

In the age group between 25 and 34, four males had primary resistance to one or more drugs, and one male patient had resistant bacilli to three drugs (with HRS pattern). Of five patients found in the age group between 35 and 44, two males and two females had streptomycin mono-resistant strains and one female with primary isoniazid mono-resistant strain. In addition, three females had *M. tuberculosis* strains resistant to one or more drugs. Moreover, all isolates of *M. tuberculosis* mono-resistant to isoniazid were found only in female patients (Figure 2).

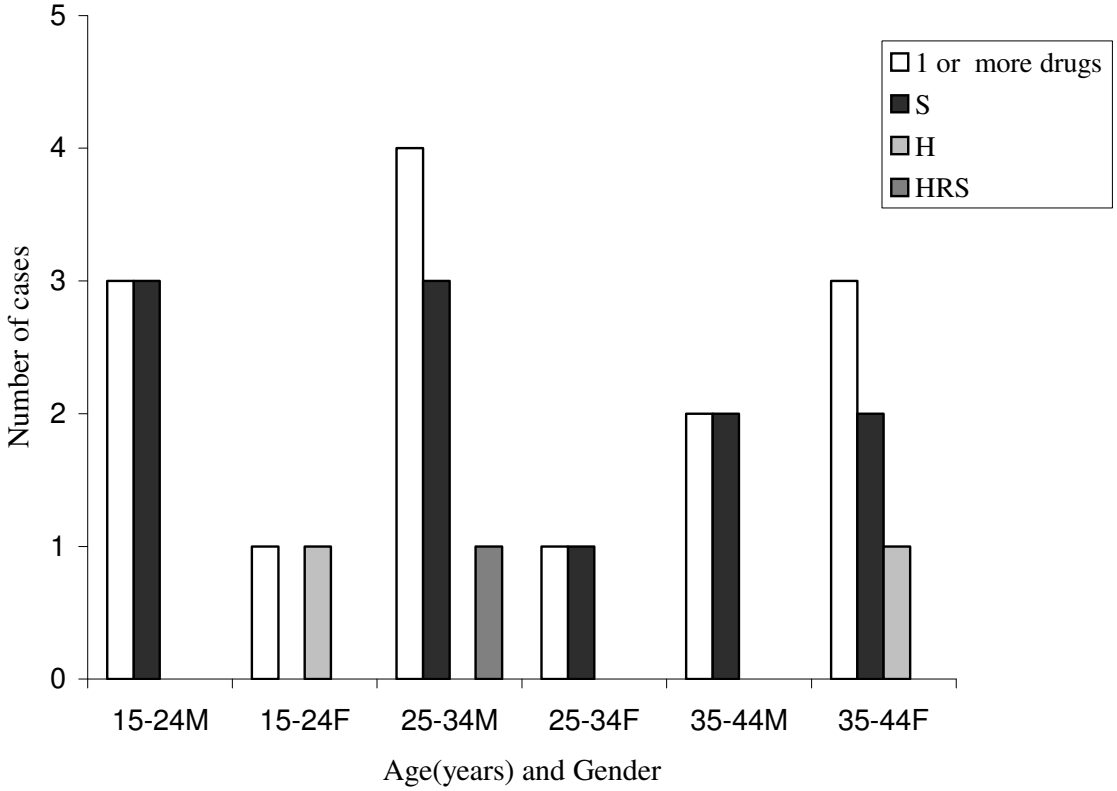

Figure 2: Distribution of primary resistance by age group and sex. M=male; F=female; S= streptomycin, H= isoniazid; R= rifampicin

The age range of TB patients that had primary resistant isolates was between 15-44 years with mean age of 29.2 years. Comparing each sex and the corresponding mean age and age range, the mean age of male patients that had primary resistant bacilli, 26 years, range 15-44 years, did markedly differ from females 31 years, age range, 20-44 years. Finally, the association

between patient's characteristics (age and gender) and primary resistance (rate) is showed in Table 4.

No correlation was found between sex and primary resistance rates. Of the fourteen patients that harbored any primary resistant strains of *M. tuberculosis*, 18.0 % of males (9/50) and 19.2 % of females (5/26) were infected by these resistant bacilli strains. Like wise, primary resistance was not associated with age and gender (P>0.05).

Table 4: Primary resistance with age and sex distribution of new PTB patients

Patient Characters (N=76)	Total cases (N=62	Sensitive cases (N=14)	Resistant cases	% resistant	P-value

Age (Years)					
12-20	23	19	4	17.4	NS (P >0.05)
21-39	38	30	8	21.1	
≥ 40	15	13	2	13.3	
Sex					
Male	50	42	9	18.0	NS (P> 0.05)
Female	26	20	5	19.2	

NS=Not significant; N= number of PTB cases

5. DISCUSSION

Information about susceptibility patterns of *M. tuberculosis* isolates against anti-tuberculosis drugs is an important aspect of tuberculosis control. Therefore, understanding drug resistance patterns in a community has an epidemiological significance in providing indicators, such as the existence and prevalence of primary and acquired drug resistance. Knowledge of the

patterns of primary resistance to commonly used anti-tuberculosis agents allows conclusions to be drawn on the therapeutic success of the drugs (WHO/IUATLD, 1997; WHO/IUATLD, 1998). Although, the link between drug resistance and TB control programme performance was noted earlier, in Ethiopia there is scarcity of information on the prevalence of anti-tuberculosis drug resistance. Because of the absence of any data on patterns of primary resistance to first-line anti-tuberculosis drugs among new pulmonary tuberculosis cases of Bahir Dar, the present study is used to generate preliminary data that contains this information.

The overall primary resistance rate of 18.4 % found in the present study among new pulmonary tuberculosis patients in Bahir Dar (Ethiopia), is comparable to that reported in Addis Ababa, 15.6 %, (Demissie *et al.*, 1997). Similar result (18.2 %) was reported in Arsi zone, Ethiopia (Gebeyehu *et al.*, 2001). However, higher resistance rate (32.5 %) than ours was demonstrated on one study in Harrar, Ethiopia (Mitike *et al.*, 1997).

Since the level of any primary resistance seen in this study is relatively higher, it suggests that *M. tuberculosis* strains resistant to any kind of anti-tuberculosis drugs are transmitted within the community of Bahir Dar.

At the time of this study, no data were obtained concerning risk factors associated with the development and acquisition of drug resistance (treatment failure, interruption, relapse) and the prevalence of acquired drug resistance from both health institutions. However, some studies conducted in the past and in recent years regarding such issues showed absence of well-maintained TB control measures in Ethiopia (Abate, 1999). For instance, a recent report by (Netto, *et al.,* 1999) on treatment success of DOTS programme in Ethiopia stated that the country scored much too low treatment success in DOTS treatment areas. This may likely mean inadequately treated cases can often develop and carry (acquire) drug resistant strains,

and are persistently infectious. Once acquired resistance develops, then the population is exposed to, and some are primarily infected with, resistant strains. Accordingly, inappropriate use of anti-tuberculosis drugs is thought to be a major risk factor for the development of drug resistance, as a consequence of TB cases mismanagement that leads to low treatment success rate (Mitchison, 1998).

In addition, Pattyn *et al*., (1979) reported high percentage of single drug resistance to isoniazid and streptomycin (46 %, to each drug) in re-treatment cases of tuberculosis in Addis Ababa. Recently, an equally high proportion of resistance to one or more drugs (50 %), with 12 % of acquired resistant MDR-TB, was reported in Addis Ababa among re-treatment cases (Abate *et al*., 1998). Therefore, the presence of high proportion of TB patients with acquired resistance indicates that these cases are the potential sources of drug resistant strains that are transmitted in a community (i.e. primary drug resistant/drug resistant strains from the outset). Thus, it follows that in a community with the rising incidence of HIV/AIDS (e.g. sub-Saharan Africa) the presence of high rate/level of acquired drug resistance may be considered as a risk factor for the widespread transmission of primary drug-resistant tuberculosis, and even for multi-drug resistant tuberculosis outbreaks (Cantwell and Binkin, 1997).

The rate of primary drug resistance varies from place to place in Ethiopia. Recent findings of two global surveillance efforts on anti-TB drug resistance showed that in several countries of the world the frequency of drug resistance including MDR-TB varies from place to place or from country to country. These common findings suggest that variations in anti-microbial resistant rates are not limited to a given geographical area, but they are also found in diverse settings of the world (WHO/IUATLD, 1997; WHO; 2000). Thus, these variations could be related to differences in the extent of exposures to selective pressures that are potentially

contributing to the development of drug-resistant bacilli. Such selective pressures could be availability of drugs, patterns of antibiotic use or differences in TB infection control. Nevertheless, there is a need for more judicious use of anti-tuberculosis agents, in settings where anti-tuberculosis drug resistance rates are high.

In this study, primary resistance to single drugs occurred in 2.6 % of the patients to isoniazid and in 14.5 % to streptomycin, which is comparable to a recent finding in Arsi zone (Ethiopia), that showed 2.3 % to isoniazid and 11.4 % to streptomycin (Gebeyehu *et al*., 2001). Similar results (2%) of isoniazid mono-resistance were reported a decade ago in Sidamo, Ethiopia (Lemma *et al*., 1989), in some African countries (Nunn and Felten, 1994; Glynn *et al*., 1995), and recently in Portugal (Antunes *et al*., 2000). Contrasting with our result, previous studies reported from different areas of Ethiopia showed high frequency of isoniazid primary mono-resistance. Three previous studies done in TB centers revealed, 12 %, 15 %, 21.4 % in 1984, 1986 and in 1997, respectively (Lemma *et al*., 1984; Wolde *et al*., 1986; Mitike *et al*., 1997) and one study reported, 8.4 % single drug resistance to isoniazid in 1997 in Addis Ababa clinics and hospitals (Demissie *et al*., 1997). This may reflect that patients coming to the health institutions are more likely to have not received prior anti-tuberculosis drug treatment as compared to the patients coming to tuberculosis centers. Nevertheless, isoniazid is an essential drug in initial treatment regimens in the country in general, and in DOTS areas in particular. Thus, the rate of single drug resistance (2.3 %) or any resistance (3.9 %) to isoniazid may be used to predict that resistant bacilli seen at present are more likely to become resistant to other anti-tuberculosis drugs or they have the potential to do so at least in the near future. Therefore, the result of the present study and that of the previous ones highlight the need for periodic monitoring of anti-tuberculosis drug resistance patterns.

Single drug resistance (14.5 %) to streptomycin found in this study was quite high. It appears to be related to the past widespread use of streptomycin in combined antibiotic treatments for infectious diseases other than tuberculosis. Over all, the tendency of primary resistance to streptomycin seems increasing in recent years. The evidence for this can be indicated from few study reports from different areas of the country that were available between the years 1984 and 2001. Over the 18-year period, single drug resistance to streptomycin increased from 2 to 5 % in 1984/89 (Lemma *et al*., 1984; Lemma *et al,* 1989) to 10 to 20 % in 1997/2001 (Demissie *et al*., 1997; Mitike *et al*., 1997; Gebeyehu *et al*., 2001). This may likely indicate, between the years 1984 to 1989 and the years 1997 to 2001, streptomycin showed a considerable increase from 5 to 10 fold.

Streptomycin and isoniazid resistance must be seriously considered since these drugs are core components of the standard and short course chemotherapy regimens against drug susceptible strains of *M. tuberculosis*. These drugs are relatively cheap drugs with a vital role in the treatment of tuberculosis in developing countries. Losing the effectiveness of these drugs may mean changing the treatment regimen to a more expensive one, and even the current standard regimen, which is considered relatively cheap, is unaffordable for many countries in the developing world.

The most significant finding in the present study was the absence of primary drug resistance to rifampicin alone and in combination with isoniazid. This may indicate that both drugs are the drug of choices in the study area (i.e. DOTS area) and active against susceptible strains of *M. tuberculosis*. This finding was consistent with the recently reported result in Arsi zone (Gebeyehu *et al*., 2001). In addition, as in most of previous studies (Demissie *et al*., 1997; Gebeyehu *et al*., 2001), no primary mono-resistance to ethambutol was detected in this study.

This also indicates that the drug ethambutol is active against all *M. tuberculosis* isolates and it remains the drug of choice in the study area. However, primary multi-drug resistance found in one patient (1.3 %) with the most common pattern of resistance to three drugs (HRS) is quite low. While low prevalence of primary MDR-TB has been found in few areas of the country, surveillance of drug resistance must be considered a priority in order to follow trends and take immediate actions. This is especially the case in countries like ours where HIV prevalence is high and where the situation could change rapidly if HIV associated MDR-TB outbreaks occur as they have in many developing and developed countries. In addition, the improvement and expansion of control activities should be promoted while limiting the availability of over-the-counter drugs. This is another measure to be considered to keep multi-drug resistant tuberculosis at very low prevalence levels (WHO, 2000).

The WHO publications on the current results of the Global Anti-tuberculosis Drug Resistance Surveillance Project stress the importance of sound TB control programmes on the success of curbing drug resistance levels and eventually, in reducing the worldwide burden of tuberculosis disease (Beccera *et al*., 2000; WHO, 2000).

In Ethiopia, the current TB control programmes involve long course chemotherapy and the short course one (NTLCP, 1997a; NTLCP, 1999). The long course chemotherapy covers 59 % of all TB cases, in areas where the DOTS programme has been not yet implemented. In the long course TB control programme, the treatment regimen consists of a two-month three drugs combination and a ten-month two drugs combination that include, 2S(TH)/10(TH) or 2S(EH)/10EH (S= streptomycin, E= ethambutol, T= thioacetazone, and H= isoniazid). Duration of treatment period takes 12 months (2 months with intensive phase and 10 months of continuation phase). The remaining 39 % covers DOTS i.e. directly observed therapy, short

course treatment strategy. DOTS regimen for new TB cases include, 2S(RHZ/6(EH) or for children of age six and below or the seriously ill, 2S(RHZ)/4(RH) or 2(RHZ)/4(RH); (P= pyrazinamide; R= rifampicin). However, the treatment protocol recommended by the World Health Organization DOTS strategy for new TB cases, 2RHZE/ 4RH or 2SHRZ/4RH (with directly observed treatment at least during the first two months of therapy) has been proved more effective than other drug combinations, for instance, 2SRHZ/6EH and long course regimens (Long and Scalcini, 1999). Moreover, streptomycin mono-resistance seems increasing in the country and its resistance has been concurrently associated with isoniazid resistance (WHO/IUATLD, 1997; Gebeyehu *et al.,* 2001). Along with our observation of a high rate of streptomycin mono-resistance, these highlights the need to use the treatment regimen 2HRZE/4HR for new PTB cases than 2SHRZ/4RH. As a result, streptomycin may gain its future treatment efficacy against tuberculosis. This is due to the fact that, use of an antibiotic in rotation certainly prevents further resistance too (Walsh, 2000). On top of this, the use of such treatment regimen has an advantage of shortening the duration of chemotherapy period from 8 months to 6 months (Olle-Goig, 2000). Therefore, in our opinion, it would seem appropriate standard short course chemotherapeutic regimen to treat new pulmonary tuberculosis patients in Ethiopia.

It is of particular interest that all isolates of *M. tuberculosis* resistant to single drug or to one or more drugs showed susceptibility to higher concentrations of each drug tested. This may reflect, regardless of the *in vitro* minimum inhibitory concentration (MIC), higher concentrations of all drugs that are well below the level achievable in serum may be active against some primary resistant strains of *M. tuberculosis.* For instance, recently isoniazid with high MIC (i.e.$\leq$ 4 µg/ml) was suggested active against some acquired multi-drug resistant strains of *M. tuberculosis* (Abate *et al*., 2001).

Finally, primary resistance in this study was observed in the young and adult age groups (between 15-44 years). This may likely indicate that a recent increase in circulating drug resistant strains might be due to a combination of reactivation of an old infection, and re-infection with new circulating strains; but this needs a more rational assessment and investigation using molecular methods like RFLP analysis (Bastian *et al*., 2000).

5.1.Limitations of the study

The present study had some limitations: First since pulmonary tuberculosis patients included in the study were not chosen on the basis of representative sampling procedures, the findings of the study can not be considered representative of the primary drug resistance situation in the general population of Bahir Dar. However, a finding based on representative study population does not change the fact that the existence of the problem (i.e. primary drug-resistant tuberculosis) in Bahir Dar. Second, to explain the results of the present finding, we cannot rule out some underreporting of primary resistance. Occasionally, the growth of drug resistant strains of *M. tuberculosis* on MB 7H10 agar medium is not sufficient (if fewer than 50 colonies) for the test to be valid. If under reporting indeed occurred, the main conclusions of this study i.e. that primary resistant strains of *M. tuberculosis* infection has occurred and can be spread among resident of Bahir Dar, would not change but instead would be reinforced.

7.CONCLUSIONS AND RECOMMENDATIONS

Tuberculosis is currently a major public health problem through out the world, where isolates of *M. tuberculosis* are resistant to first-line anti-tuberculosis drugs. This means that inexpensive antibiotics that would decrease the spread of TB by decreasing the bacilli burden are no longer effective.

It is the use of anti-tuberculosis drugs for prophylactic or therapeutic purposes against

TB or other infectious diseases that provides selective pressure favoring over-growth of resistant bacilli. Indeed, inappropriate drug use or treatment could lead to a risk that these drugs become less effective in treating active tuberculosis. As the pool of patients excreting drug resistant bacilli expands (i.e. acquired resistant cases as a result of the recent past poor infection control), there is an increasing risk of transmission of the bacilli to healthy individuals who can be infected with resistant strains and develop drug resistant disease (TB) from the outset i.e. primary resistance. The prevalence (level) of primary resistance in a community is, therefore, an excellent long-term indicator of the overall quality of TB treatment and control.

In Bahir Dar, the overall primary resistance rate for any drug is 18.4 %, and any primary resistance to streptomycin is 17.5 %; for isoniazid it is 3.9 %; and for primary multi-drug resistance, it is 1.3 %, all are cause for concern, at least in the near future. Although the rate for any primary resistance is moderate, *M. tuberculosis* strains resistant to one drug, or more than one drug are likely to become resistant to others. Hence, a rifampicin-based 6-month chemotherapy devoid of streptomycin seems reasonable in decreasing the pressure favoring the spread of *M. tuberculosis* strains that were already resistant to any kind of anti-tuberculosis drug.

Single drug resistance either to rifampicin, or to ethambutol alone is nil. This makes both drugs active against all strains of *M. tuberculosis* and the drug of choices for the ongoing DOTS tuberculosis treatment programme of the study area. This same conclusion can also be applied to combinations of rifampicin and isoniazid, as no primary resistance was found regarding a combination of both drugs.

In general, the present study highlights the need to periodic surveys to monitor anti-TB drug-resistance patterns/levels in Bahir Dar. Failure of monitoring and tracking changes in drug-resistance will fuel further resistance. Unfortunately, once resistance appears and increases, it is likely to decline slowly, if at all. In addition, there are no counter-selective measures against drug/multi-drug resistant tuberculosis, or if available, second-line drugs are more expensive and less effective. Therefore, periodic surveys are important to follow trends of drug resistance levels and to take immediate actions that could improve the TB control programme of a given setting.

To this end, based on the present information obtained on primary drug resistance patterns of *M. tuberculosis* strains among new pulmonary tuberculosis patients of Bahir Dar, and other similar studies that have been conducted previously in the country, the following preliminary tuberculosis control/management measures that would contribute to containing the existing drug resistant tuberculosis situation and improving the tuberculosis control programme performance of the study area/ country are suggested as recommendations.

- In this era of increasing anti-tuberculosis drug resistance, there is clearly a need to continue with studies in pattern of drug resistance in Bair Dar and other settings of the country in order to follow changes (trends) of the problem and to take immediate actions with time.

- Better programme management and enhanced public health efforts that will prioritize an increase in cure rates of new TB cases (i.e. that will reduce transmission of TB and

contain low level drug resistance) using the most appropriate and effective standard short course treatment regimens are needed.

- Tuberculosis case detection rate, cure rate as well as the prevalence of acquired drug resistance should be thoroughly studied as indicators of programme performance.

- The epidemiological association between drug resistance and HIV/AIDS, and comparatively young age (15-44) should be studied, in order to understand risk factors and the dynamics of drug resistant tuberculosis transmission.
- Public health education/ public awareness directed towards the prevention and control of TB/drug resistant tuberculosis transmission should be considered.

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Annex I. Sputum specimen collection Form

Ser. No.	Sputum collection Date	Code/Ref No.	Age	Sex	Address		
					In Bahir Dar town	Around Bahir Dar town	Outside Bahir Dar area

Annex II. A *Mycobacterium* Growth Chart

Serial No.	Isolate Lab. Code No.	AFB smear result	Date of Inoculation	Weeks								Remarks
				1	2	3	4	5	6	7	8	

Annex III. Drug susceptibility test result recording form

Date of inoculation	Date of interpret-ations	Strain No.	Number of colonies grown on drug free and drug containing media										Remark (final result, contamination, etc.)
			10^{-1} control	10^{-3} control	H-1	H-0.2	R-1	S-10	S-2	E-10	E-5	TCH-2	

H= isoniazid, R=rifampicin, S=streptomycin, E= ethambutol, TCH= thiophen-2-carboxylic acid hydrazide. Numbers in front of the abbreviations of each drug indicate µg/ml of drug concentration used.