CHAPTER I

1. INTRODUCTION

Tuberculosis (TB) constitutes a major public health problem in most developing countries of the world. It accounts for the largest burden of mortality due to any infectious agent worldwide. The incidence of TB rose so rapidly over a number of years that World Health Organization (WHO) was compelled to declare it a global emergency in April 1993, the first declaration of this sort ever (WHO, 1993). Tuberculosis in man is caused predominantly by *Mycobacterium tuberculosis* and occasionally by *Mycobacterium bovis* and *Mycobacterium africanum*. These organisms are also known as tubercle bacilli because they cause lesions called tubercle and or as acid fast bacilli (AFB) (once stained by hot carbol fuchsin, they resist decolorisation by dilute mineral acids and are therefore referred to as acid fast bacilli).

It is estimated that up to one-third of the world's population is infected with the TB organism. There were 8.8 million new TB cases in 2005 and 80% of them in 22 countries. 1.6 million people died of TB in 2005, equal to an estimated 4400 deaths a day. TB is a leading killer among HIV-infected people with weakened immune systems; about 200,000 people living with HIV/AIDS die from TB every year, most of then being in Africa. Similarly, 450,000 new multi-drug resistant TB (MDR-TB) cases are estimated to occur every year (WHO, 2007).

Tuberculosis is an immense problem in Nepal, causing great suffering and death. In Nepal, about 45% of the total population is infected with TB, of which 60% are adult. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease and 5,000 to 7,000 people die from TB. The latest survey conducted in 2001/2002 showed MDR-TB of 1.30%. In 2002, 2.4% of TB patients were infected with HIV (NTC, 2007).

Tuberculosis is a socio-medical problem (STC, 2001). Diagnosis is performed clinically, radiologically and bacteriologically. Demonstration of bacillus in the lesions by direct microscopy either by Ziehl-Neelsen staining or by fluorochrome staining allows highly accurate diagnosis. Radiological examination of TB is done by chest-X-ray. Radiological examination can be compatible with diagnosis of pulmonary tuberculosis where smears are repeatedly negative. Examination by bacteriological culture provided the definitive diagnosis (WHO, 1998). Different biochemical tests such as niacin test, nitrate test, catalase test etc. confirms the identification in culture (Kent *et al.*, 1985). Culture also provides the necessary material for drug susceptibility testing. There are also various molecular techniques which help in identification of mycobacteria, such as Polymerase chain reaction (PCR), Ligase chain reaction (LCR), Gas liquid chromatography (GLC), High performance liquid chromatography (HPLC) etc. (Forbes *et al.*, 2002).

Susceptibility testing is used to determine the resistance of strains isolated before the commencement of treatment (initial or primary resistance); or to discover whether resistance has arisen during treatment (secondary resistance) (Inderlied *et al.,* 2003). Different conventional methods (the resistance ratio, the proportion, the absolute concentration), radiometric method and other different newer methods are used for determining antimicrobial susceptibility pattern. The most extensively used being the resistance ratio and the proportion methods. The resistance ratio method compares the resistance of unknown strains of tubercle bacilli (test organism) with that of a standard laboratory strain ($H_{37}Rv$); whereas in the proportion method the degrees of growth of test organism in drug containing media and drug free media are compared (Rieder *et al.,* 1998).

The accurate susceptibility testing method is very important to determine the exact susceptibility pattern of *M. tuberculosis* and hence this study is undertaken to compare the two methods (resistance ratio and proportion method) for susceptibility testing of *M. tuberculosis* and to find out the agreement rate between them.

CHAPTER II

2. OBJECTIVES OF THE STUDY

2.1 General objective

Comparative evaluation of two different *in vitro* anti-tubercular susceptibility testing methods in Nepalese context.

2.2 Specific objectives

1. Evaluation of anti-tubercular susceptibility pattern of mycobacterium isolated from patients visiting National Tuberculosis Centre using Resistance ratio method.
2. Evaluation of anti-tubercular susceptibility pattern of mycobacterium isolated from patients visiting National Tuberculosis Centre using Proportion method.
3. Comparison of above two methods.

CHAPTER III

3. LITERATURE REVIEW

3.1 Tuberculosis (Definition)

Tuberculosis is a chronic bacterial infection caused by *Mycobacterium tuberculosis* (and occasionally by *Mycobacterium bovis and Mycobacterium africanum)* and characterized by the formation of granuloma in infected tissue as a result of cell-mediated hypersensitivity. This infectious disease primarily affects lungs causing pulmonary tuberculosis but may also cause extra-pulmonary tuberculosis affecting intestine, meninges, bones and joints, lymph node, skin and other parts of the body (Park, 2005). The commonest symptoms of pulmonary tuberculosis are cough for three weeks or more, fever, lethargy, lassitude, decreased appetite and weight loss and haemoptysis. In extra-pulmonary TB, symptoms depend on the organs involved (WHO, 2002).

TB is often the first disease to occur in the AIDS patients, even before any of opportunistic disease appears, and it is generally more intractable than in non-AIDS patients (Alcamo, 1995).

3.2 History

In the past tuberculosis has been referred to as the “great white scourge” and by John Bunyan, as “the captain of all of these men of death”. The clinical features of both pulmonary and spinal tuberculosis were well described by Hippocrates in about 400 BC. Accounts of the disease appeared in the Vedas and other ancient Hindu texts, in which it was sometimes, termed “Rajayakshma” (the king of disease) and it afflicted Neolithic man and pre Columbian Amerindians (Grange, 1990). Hippocrates gave an excellent clinical description of the disease called “pthisis”, a Greek word which mean “to consume to spit” and “to waste away” (Grange, 1996). The Dutch physician, Franciscus Sylvius (1614-1672) deduced from autopsies that tuberculosis characterized by the formation of nodules, which he named “tubercles” (Lowell *et al.*, 1969).

The transmissible nature of tuberculosis was clearly established by Jean-Antonie Villemin, a French military doctor. J.L Schonlein is credited to have named the disease "tuberculosis" (Rosenblatt, 1973). Villemin's prediction that the causative agent of tuberculosis would be isolated was realized in 1882 when Robert Koch succeeded in culturing it on inspissated serum. Thus, Koch announced discovery of the tubercle bacillus in 24th March 1882. In addition to culturing the causative organism, Koch succeeded in staining it by treatment with an alkaline solution of Methylene blue for 24 hours. The acid fast nature of the organism was discovered by Ehrlich. He improved the technique by using hot solution of the aryl methane dye fuchsin. And the present method of acid fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence the name Ziehl-Neelsen staining technique (ZN staining) was established.

Though the disease has been identified earlier, the modern era of tuberculosis treatment began only in 1946 with the advent of Streptomycin and in 1952 with development of Isoniazid hydrochloride (INH). Since then the modalities of treatment regimens were constantly revised and updated.

3.3 Epidemiology

All members belonging to Mycobacterium Tuberculosis Complex (MTC) cause tuberculosis infections. *Mycobacterium tuberculosis* is the cause of most cases of human tuberculosis, particularly in developed countries. An estimated 2 billion persons, one-third of the world's population, are infected with *Mycobacterium tuberculosis.* Of great concern is the emergence of epidemic multi-drug resistance strains of *Mycobacterium tuberculosis* (Forbes *et al.,* 2002).

3.3.1 Global situation

TB is a disease of poverty; affecting mostly young adults in their most productive years; the vast majority of TB deaths are in the developing world, with more than half of all

deaths occurring in Asia. TB is contagious and spread through the air; if not treated; each person with Active TB infects on average 10-15 people every year, 1 in 10 people infected with TB bacilli will become sick with active TB in their life time; people with HIV are at much greater risk (WHO, 2007).

There were 8.8 million new TB cases in 2005 and 80% of them in 22 countries. 1.6 million people died of TB in 2005, equal to an estimated 4400 deaths a day. TB is a leading killer among HIV-infected people with weakened immune systems; about 200,000 people living with HIV/AIDS die from TB every year, most of them being in Africa. TB is a worldwide pandemic; although the highest rates per capita are in Africa (28% of all TB cases), half of all new cases are in 6 Asian countries (Bangladesh, China, India, Indonesia, Pakistan, Philippines). TB annual incidence rates are now stable or falling in all six WHO regions and have peaked globally, however, the total number of cases is still rising in the African, Eastern Mediterranean and South-East Asia Regions (WHO, 2007).

3.3.2 Situation in Nepal

TB is a major public health problem in Nepal. About 45% of the total population is infected with TB, of which 60% are adult. Every year, 40,000 people develop active TB, of whom 20,000 are able to spread the disease to others. Although introduction of treatment by Directly Observed Treatment Short-course (DOTS) has already reduced the numbers of deaths; however 5000-7000 people still continue to die each year from TB (NTC, 2007).

In Nepal, majority of cases are in rural areas where more than 90% of population resides. The annual rate of infection is estimated at about 3%. In hilly area it is about 1.5%, in terai area is about 2.5%, in urban area it is about 4% and in mountain area is less than 1%.

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3.4 Bacteriology

3.4.1 Etiological agents

The genus Mycobacterium is the only genus in the family Mycobacteriaceae. Currently, there are 71 recognized or proposed species in the genus Mycobacterium (Forbes *et al.*, 2002). It is convenient to divide mycobacteria of clinical interests into MTC and mycobacteria other than tuberculosis bacilli (MOTT). MTC is associated with tuberculosis which includes *Mycobacterium tuberculosis, M. bovis, M. africanum, M. microti* and BCG. And MOTT may be associated with other human disease. Several other collective names given to MOTT are atypical, anonymous, non-tuberculous, tuberculoid, opportunistic, environmental bacilli (Collee *et al.,* 1996).

Most human TB is caused by *M. tuberculosis* but some cases are due to the *M. bovis,* which is principal cause of tuberculosis in the cattle and many other animals. But *M. bovis* rarely cause diseases in the area of the world where animal husbandry includes TB screening and milk pasteurization. *M. microti* is a pathogen of voles and other small animals. *M. africanum* believed to represent a transitional organism between *M. bovis and M. tuberculosis* and also cause human tuberculosis and mainly found in equatorial Africa (Chakraborty, 2003).

MOTT exists as saprophyte of soil and water and occasionally causes opportunistic disease in human. Infection caused by this organism is known as mycobacterioses. Four main types of diseases are caused by MOTT. They are skin lesions followed by traumatic inoculation of bacteria, localized lymphadenitis, and tuberculosis like pulmonary lesions and disseminated diseases (Chakraborty, 2003).

Three main types of skin lesions are caused by MOTT bacteria. Post injection abscesses are caused by rapidly growing pathogens mainly *M. chelonei* and *M. fortitum*, swimming pool granuloma (also called fish tank granuloma) caused by *M. marinum* and buruli ulcers caused by *M. ulcerans*. Pulmonary disease is most frequently caused by *M.*

kansasii. Most cases of disseminated disease are caused by *M. aviumintracellularae* and this species is now a well recognized cause of secondary disease in AIDS victims.

Though MOTT infections have been widely reported from all over the world, in Nepal reliable data on MOTT infection are not available due to lack of culture facilities and a proper reporting system. In high TB prevalence countries more than 85% of disease is due to *M. tuberculosis* and other mycobacteria rarely are responsible for clinical disease (WHO, 1998).

It is clear that the mycobacteria are the cause of very important group of infectious diseases of man (Grange, 1998).

3.4.2 General characteristics and morphology

Organisms belonging to the genus mycobacterium are very thin, slender, complex, unicellular organisms with a wide range of antigenic determinants (Collee *et al.,* 1996). Size varies from 0.2 to 0.4 × 2 to 10 μm (Forbes *et al.,* 2002). Mycobacteria are acid-alcohol fast, non-motile, non-sporing, weakly gram positive, aerobic or micro-aerophilic, straight or slightly curved rod-shaped bacteria. Some mycobacteria display cocco-bacillary, filamentous or branched forms and some produce yellow to orange pigment in the dark or after exposure to light (Good *et al.,* 1998).

The typical cell morphology of *M. tuberculosis* as seen in acid fast stains is a thin, straight or slightly curved (bacillus) rods measuring of about 0.3 to 0.6 by 1 to 4 μm deeply stained (strongly acid fast), with a distinct beaded appearance. They are non-motile, non-sporing and have no capsules. They are gram positive but many species stain poorly with this stain even after prolonged staining, because of the characteristics of the cell wall, which is rich in chemically diverse lipids (upto 60% of the cell wall). The thickness of the cell wall is due to the presence of long chain fatty acids (mycolic acids) which form a thick palisade. It is β-hydroxy fatty acid linked covalently to

murein. Hence the slow growth results from inability to transport nutrients rapidly across the wax layer (Collee *et al.*, 1996).

3.4.3 Cultural characteristics

Tubercle bacilli are obligate aerobes and will not grow in the absence of oxygen, even a moderate reduction in the oxygen tension result in an appreciable decrease in the metabolism of the bacilli. The bacilli grow slowly, the generation time *in vitro* being 14-15 hours. Colonies appear only in about two weeks and sometimes may be delayed up to 6 to 8 weeks. Optimum temperature is 37°C and growth does not occur below 25°C or above 40°C. Optimum p^H is 6.4 to 7.0 and grows only in especially enriched media containing egg, asparagines, potatoes, serum and meat extract (Forbes *et al.*, 2002).

3.5 Pathogenesis

The clinical manifestation and eventual outcome of TB-as of all infectious processes depend on the virulence of the pathogen and the nature of the host's immune responses (Grange, 1998).

3.5.1 Mode of infection and transmission

Most of cases primary tuberculosis (PTB) begins in the lung as a result of inhalation of bacilli. Transmission is by the respiratory route. When air borne particles less than 3 mm in size are inhaled they are not trapped in the nose but may reach the alveoli. One air borne particle 1-3 µm contains 1-10 bacilli. Patients who excrete 10,000 or more tubercle bacilli per ml. of sputum are the main source of infection to others.

Chakraborty (2003) describes three modes of infection:

- Infection in man is transmitted mainly by inhalation of bacilli in moist droplets of respiratory secretions from the open pulmonary tuberculosis.

- Occasionally infection occurs by ingestion of infected milk. *M. bovis* in the past was responsible for majority of cases of intestinal, glandular and bone tuberculosis in west due to drinking of infected milk.
- In certain occupations where workers are exposed to inhalation of stone or metal dust such as quarry man, plasters and miners, incidence of TB is high.

It is well established that patients with sputum that is positive on direct smear examination are the principal sources of infection. Smear negative patients, whether culture-positive or not, are of very low infectivity (Grange, 1998). Two factors determine an individual risk of exposure; the concentration of droplet nuclei in contaminated air and the length of time breathing that air. The risk depends greatly on the closeness of contact as well as the infectiousness of the source case. All children with household exposure to adults with active TB have a high risk of contracting the infection and disease. Risk specially increases in crowded and poorly ventilated areas.

Thus, TB is spread by airborne infectious droplet nuclei. They are expelled in the air with coughing, sneezing, singing, laughing, talking etc. and remain suspended in the air for many hours. Coughing generates the largest number of droplet of all sizes (Park, 2005). One cough produces 3000 to 5000 droplet nuclei. Transmission generally occurs indoors, where droplet nuclei can stay in the air for a long time. Ventilation removes droplet nuclei. Direct sunlight quickly kills tubercle bacilli, but they can survive in the dark for several hours.

3.5.2 Predisposing factors in the development of TB

The incidence of tuberculosis varies according to ethnic origins and socioeconomic status within a community; the major determining factor is overcrowding (Grange, 1998). Predisposing factors for progression of disease are old age, alcoholism, diabetes neoplastic disease, malnutrition, immunosuppressive drugs, stress and drug induced, congenital or acquired immunodeficiency including HIV infection. Certain groups have high risk for development of TB and they are refugees, physical and psychological

stress, nursing home residents and the impoverished. Also the vulnerable groups or the individuals more susceptible to the disease include persons under conditions like, poorly controlled diabetes mellitus, chronic lung disease (bronchitis) and silicosis, cancer, advance kidney disease, malnutrition, alcoholism, disease for which steroid therapy is prescribed; heavy smokers, elderly, low income groups, intravenous drug users, living and attending drug treatment centers, hospital, nursing homes.

As immunity wares, through aging or immune suppression, the dormant bacteria reactivate, causing an outbreak of disease often many decades after the initial infection (Collee *et al.*, 1996). Thus, the incidence of disease in a community may be affected by many factors, including the density of the population, the extent of over crowding and the general standard of living and health care (Grange, 1998). The risk of tuberculosis is greater in areas of residence characterized by crowding, poverty and lower education.

3.5.3 Virulence factors of tubercle bacilli

The virulence of tubercle bacilli appears to be related to their ability to survive within macrophages. Tubercle bacilli inhibit the fusion of the phagosome with lysosomes, by a mechanism that is poorly understood: the bacilli secrete several compounds (ammonium ions, polyglutamic acid, cyclic AMP and sulpholipids) that affect cell membranes, but their role *in vivo* is uncertain. It has been suggested that the alteration in membrane function may isolate the bacilli from nutrients as well as from the lysosomal contents, and this may be the mechanism of bacterial dormancy and persistence (Grange, 1998).

Mycobacterial virulence factors can have both direct and indirect effects on host cells and can ultimately lead to tissue destruction and disease. Direct cell-cell interactions, including attachment, invasion, and intracellular multiplication and indirect interactions, through secreted bacterial factors such as hemolysin and cytotoxin, can cause lysis of the host cells. In addition, lipoarabinomannans (LAMs), heat shock proteins, and Mycobacterial products can stimulate host cell to produce inflammatory products or cytokines that can amplify tissue damage in the host (Quinn, Newmann et al. 1996). The

virulence of tubercle bacilli is due to its resistance to the production of toxic substances (Grange, 1990). The virulence of tubercle bacilli appears to be related to their ability to survive within macrophages and the organisms exhibit long periods of metabolic inactivity. Some virulence factors of *M. tuberculosis* are cord factor, pthiocerol dimicocerosate (Kulkarni, 1998). Mechanisms hypothesized for how tubercle bacilli avoid being killed by macrophages include:

- Prevention of an oxidative burst in phagocytosing cells.
- Inhibition of phagosome-lysosome fusion.
- Resistance to lysosomal enzymes such as lysozyme.
- Secretion of inhibitors or inactivators of bactericidal agents such as peroxide or oxygen radicals.
- Exudation of lipids or capsules to block the assess of bactericidal agents to the targets and
- Escape from the phagosome into the cytoplasm

Several bacterial components have been implicated in these various hypotheses and, by analogy with other bacterial pathogens, are suspected to be virulence factors of Mycobacteria. For example, phenolic glycolipids can act as scavengers of free radicals and the Mycolic acid layer can act as a hydrophobic barrier to bactericidal agents.

3.5.4 Immunity to tuberculosis

3.5.4.1 Innate immunity

Very little is known about the mechanism of the naturally occurring resistance of various mammalian species, including man, to the different types of *M. tuberculosis* (Grange, 1998). The anatomy of the nasal passages, the whirling air current which tends to deposit particles on the sides, the mucus secretions, the lysozymes and the cough reflex offer physical obstacles to the entry of the mycobacteria through the air passages to the lungs (Wilson and Miles, 1957). Various physiological factors also prevent the

infection, some of which are anti-mycobacterial substances in normal urine (Dold, 1977). Kidney tissue is known to contain spermine which has a powerful bacteriostatic action on tubercle bacilli when activated by spermine oxidase. Serum antibodies such as agglutinins, precipitins and complement fixing serum antibodies are active against antigens of tubercle bacilli (Chandrasekhar *et al.,* 1981).

3.5.4.2 Specific cell-mediated immunity

The protective immune response in TB is by "cell-mediated immunity". It has been widely accepted that specific immunity to TB is due to presentation of mycobacterial antigen to helper T-cells; proliferation of these cells; the subsequent production of macrophage-activating lymphokines; and the destruction of mycobacteria by the activated macrophages (Grange, 1998). On the other hand, acquired immunity to TB is characterized by increased resistance to the intracellular pathogens manifested by the cells after infection or vaccination (Chandrasekhar *et al.*, 1981).

Elimination of *M. tuberculosis* infection mainly depends on the success of the interaction between infected macrophages and T-lymphocytes. CD4+ T-cells exert their protective effect by the production of cytokines, primarily gamma-interferon, after stimulation with mycobacterial antigens. Other T-cell subsets, like CD8+ T-cells, are likely to contribute as well, by secreting cytokines and lysing infected cells.

3.5.4.3 The role of the macrophage

It is generally assumed that the activated macrophage can kill tubercle bacilli, but this has been difficult to prove experimentally. When *M. tuberculosis* is inhaled into the lung, they are engulfed by alveolar macrophages, which perform three important functions. First, they produce proteolytic enzymes and other metabolites, which exhibit mycobactericidal effects. Second, macrophages process and present mycobacterial antigens to T-lymphocytes, including CD4 and CD8 T-lymphocytes, which are central to acquired resistance to *M. tuberculosis.* Third, macrophages produce a characteristic

pattern of soluble mediators (cytokines) in response to *M. tuberculosis* that have the potential to exert potent immunoregulatory effects and to mediate to many of the clinical manifestations to tuberculosis.

Macrophages not only destroy bacteria, or inhibit their growth; they also secrete a wide range of immunologically relevant proteins, some of which are concerned in the pathogenesis of TB. Activated macrophages secrete proteases that liquefy caseous material with post-primary pulmonary lesions and thereby play role in cavity formation. They also secrete tumor necrosis factor (TNF) which may be responsible for both protective and pathologic effects in *M. tuberculosis* infection. Local release of TNF at the site of disease contributes to granuloma formation, control of infection and mycobacterial elimination. Excessive local production of TNF may cause extensive tissue necrosis seen in post-primary disease. This factor is identical to cachectin, which causes weight loss and is pyrogenic. It might therefore be the cause of fever and wasting characteristic of advanced TB (Grange, 1998).

3.5.5 Types of tuberculosis

3.5.5.1 Pulmonary tuberculosis

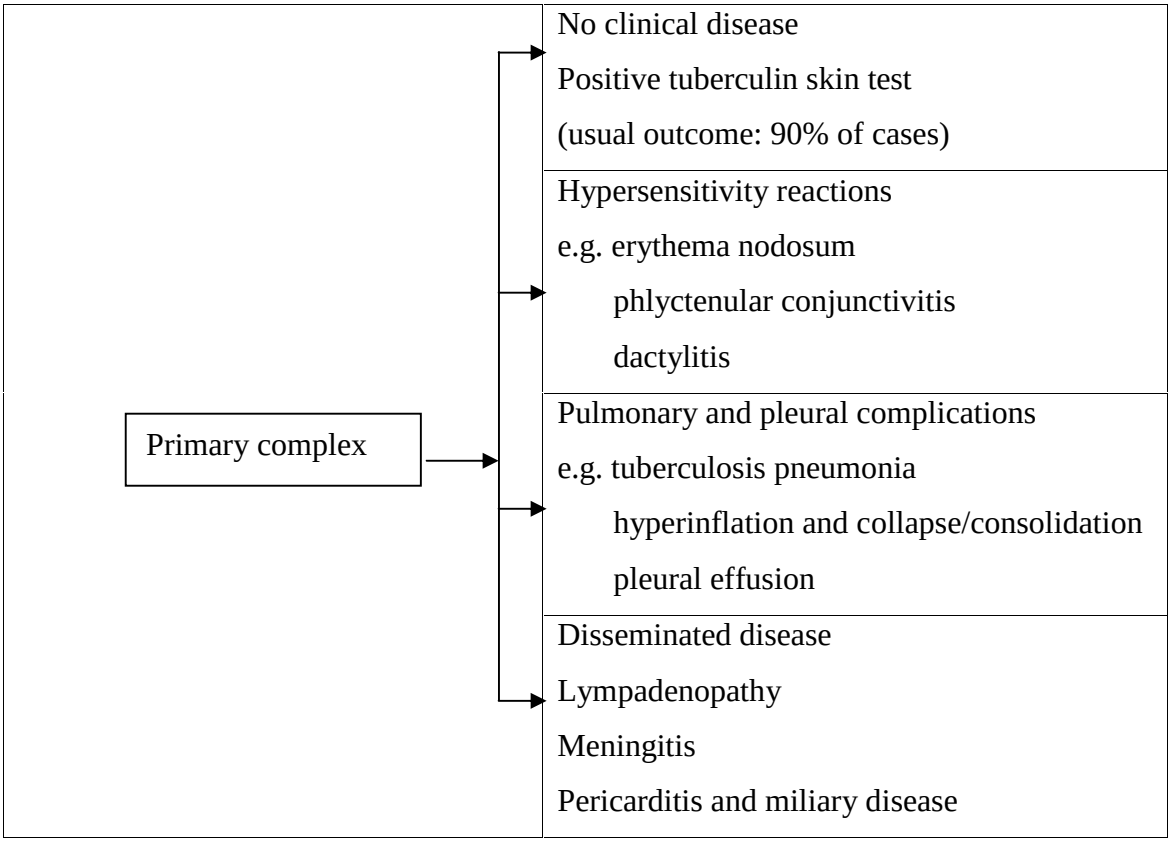
Pulmonary tuberculosis refers to disease involving the lung parenchyma. Because the lung is the usual site of primary lesion and the principal organ involved, pulmonary tuberculosis is the most common form of infection.

3.5.5.1.1 Primary infection

Primary infection occurs in people who have not had any previous exposure to tubercle bacilli. Droplet nuclei, which are inhaled into the lungs, are so small that they avoid the mucocilliary defences of the bronchi and lodge in the terminal alveoli of the lungs. Infection begins with multiplication of tubercle bacilli in the lungs. The resulting lesion is the Ghon focus. Lymphatics drain the bacilli to the hilar lymph nodes. The Ghon focus and the related hilar lymphadenopathy form the primary complex. Bacilli may

spread in the blood from the primary complex throughout the body. The immune response (delayed hypersensitivity and cellular immunity) develops about 4-6 weeks after the primary infection. The size of the infecting dose of bacilli and the strength of the immune response determine what happens next. In most cases, the immune response stops the multiplication of bacilli. However, the few dormant bacilli may persist. A positive tuberculin skin test would be the only evidence of infection. In a few cases the immune response is not strong enough to prevent multiplication of bacilli, and disease occurs within a few months (WHO, 2004).

Table 1. Outcomes of primary infection



3.5.5.1.2 Post-primary tuberculosis

Post-primary TB occurs after a latent period of months or years following primary infection. It may occur either by reactivation of the dormant tubercle bacilli acquired

from a primary infection or by reinfection. Reactivation means that dormant bacilli persisting in tissues for months or years after primary infection starts to multiply. This may be in response to trigger, such as weakening of the immune system by HIV infection. Reinfection means a repeat infection in a person who has previously had a primary infection.

The immune response of the patient results in the pathological lesion that is characteristically localized, often with extensive tissue destruction and cavitations. Post-primary TB usually affects the lungs but can involve any part of the body. The characteristics features of the post-primary PTB are the following: extensive lung destruction with cavitation; positive sputum smear; upper lobe involvement, usually no intrathoracic lymphadenopathy. Patients with these lesions are the main transmitters of infection in the community (WHO, 2004).

3.5.5.2 Extra pulmonary tuberculosis

TB can affect any organ and tissue of the body. Most, if not all, extra pulmonary lesions result by haematogenous spread of the organism from a primary focus which is not always detected (Chakraborty, 2003). Common forms of extra pulmonary TB include the following: lymphadenopathy, pleural effusion TB, meningitis, miliary, intestinal, bone, urogenital, skin and eye TB (NTC, 1997). Patients usually present with constitutional features (fever, night sweats, weight loss) and local features related to the site of diseases.

3.6. Diagnosis of pulmonary tuberculosis

The diagnosis of TB refers to the recognition of an active case i.e. a patient with a symptomatic disease due to lesion caused by *M. tuberculosis.*

3.6.1 Clinical diagnosis of pulmonary TB

The symptoms of PTB in adult include chronic cough with the production of mucopurulent sputum which may contain blood (haemoptysis), loss of weight, fever,

tiredness, chest pain, anemia, and night sweats (during sleep). The disease in children is not easy to diagnose since there is hardly any productive cough. The symptoms are usually weight loss and enlargement of the lymph glands which may cause the obstruction of the bronchi and emphysema.

3.6.2 Radiological diagnosis of pulmonary TB

Chest radiology is often used for suspected TB patients, where and when available, as evidence of TB disease. Even very experienced doctors looking at chest X-ray find it extremely difficult to differentiate between active TB; other diseases and old healed TB. If X-ray is available, diagnosis of TB should not be made on a single abnormal X-ray (except for miliary TB). Its low sensitivity and specificity render it unreliable (NTC, 1997).

3.6.3 Laboratory diagnosis of pulmonary TB

The highest priority for TB control is the identification and cure of infectious cases i.e. patients with sputum smears positive PTB. Therefore, all patients with clinical features suggestive of PTB must submit sputum for diagnostic sputum smear microscopy (WHO, 2004). Failure to diagnose TB may not only delay appropriate therapy, but may lead to the spread of TB in the community or the health care setting. And the diagnosis of TB is based on the detection of AFB in clinical specimens.

3.6.3.1 Specimen collection and transport

The most usual specimen for diagnosis of PTB is sputum. But if none is produced, bronchial washing, brushing or biopsies, laryngeal swabs and early morning gastric aspirates (to harvest any bacilli swallowed overnight) may be examined (Bass *et al.*, 1990). Specimens are collected in sterile, leak-proof, disposable, and appropriately labeled containers and placed into bags to contain leakage should it occur (Forbes *et al.*, 2002). The quality and the quantity of the sputum as well as the design of the container plays vital role in the diagnosis of etiological agent.

A PTB suspect should submit three sputum samples for microscopy. The chances of finding TB bacilli are greater with three samples than with two samples or one sample. Secretions build up in the airways overnight. So an early morning sputum sample is more likely to contain TB bacilli than one taken later in the day (WHO, 2004). Sputum specimen must be free of food particles, residues and other extraneous matter. Saliva and nasal secretions are not to be collected nor is the patient to use oral antiseptics during the period of collection. The aerosol (Saline) induction procedure can best be done on ambulatory patients who are able to follow instructions. Aerosol induced sputum have been collected from young children. Sputum specimens delivered promptly to the laboratory and refrigerated if processing is delayed. Gastric lavage is used to collect sputum from patients who may have swallowed sputum during the night. The procedure is limited to senile, non-ambulatory patients, children younger than three years of age, and patients who fail top produce sputum by aerosol induction (Forbes *et al.,* 2002).

Collection of appropriate specimens and their transportation are critical consideration, since any results the laboratory generates will be limited by the quality of the specimens and its condition on arrival in the laboratory. Specimens should be sent promptly to the laboratory to avoid being overgrown with organisms other than mycobacteria. If delay is unavoidable, specimens should be stored in the refrigerator. Specimens sent through the post in warm weather should be packed in dry ice or with an ice pack (Collee *et al.,* 1996).

3.6.3.2 Sputum evaluation and macroscopic examination

A good sputum specimen consists of recently discharged material from the bronchial tree, with minimal amount of oral or nasal material (Inderlied *et al.,* 2003). Satisfactory quality implies the presence of Mucoid or mucopurulent material and is of greater significance than volume. Ideally, a sputum specimen should have a volume of 3-5 ml, although smaller quantities are acceptable, if the quality is satisfactory.

Macroscopic examination includes the observation of the colour and appearance of sputum sample.

When sputum specimen contains mostly saliva, the specimen is reported as “unsuitable for microbiological examination” and should request for another specimen (Cheesbrough, 2002).

3.6.3.3 Microscopic examination

Microscopic examination of sputum is of great value in making the diagnosis of PTB. The main values of AFB microscopy for diagnosis lie in its speed and extremely high specificity, while the main disadvantage is said to be its low sensitivity. Specificity is very high, probably over 99% in many high prevalence settings using trained personals and good microscopes and sensitivity ranges from about 25% to 75%. The minimum number of AFB necessary to produce a positive smear result has been estimated to be between 5000 and 10000 per milliliter of sputum (WHO, 1998). But microscopy can not diagnose between live and died AFB, so that some patients excreting non-viable bacilli at the end of treatment may be roughly considered as failure-cases (Deun *et al.,* 2001).
Mycobacteria are “acid and alcohol- fast bacilli” (AAFB), often shortened to AFB. The waxy coat of mycobacteria retains an aniline dye (e.g. carbol fuchsin) even after decolorization with acid and alcohol (WHO, 2004). At present time, two methods ZN and fluorescent method are generally used to observe the AFB by microscopy.

The standard ZN method requires heating the carbol fuchsin-covered slide with a flaming torch for several minute without boiling to stain the organisms and mineral acids to decolorize the background. The background is then counter-stained with another dye such as malachite green or Methylene blue to give red AFB against a green or blue background (Collee *et al.,* 1996).

Fluorescent staining uses rhodamine-auramine stain for the detection of AFB. Fluorescent dyes such as the auramine-phenol stain work in exactly the same way as the

ZN stain but produce golden yellow brightly fluorescing bacilli against a dark background. Though it requires special fluorescence microscope for its detection, it yields better identification of the bacilli. Also that larger number of samples can be observed in limited time without much stress. If the daily examination exceeds 50, fluorescent method is to be preferred (WHO, 1998).

Fundamentally, the principle is same in both the methods, i.e. to demonstrate that the bacilli retain the stains after decolorization with acids and alcohol. In the preparation of the smears, certain important points has to be remembered viz. use of new slides, careful selection of purulent or necrotic material and preparation of a thin and evenly spread smear, and fixing by passing through Bunsen flame.

3.6.3.4 Culture of sputum sample

TB can be definitely diagnosed by isolating the causative organisms in pure culture. So, culture remains the "gold standard" for diagnosis of TB. It is most sensitive than AFB staining method and can reliably find mycobacteria when they are present in the concentration of about 10^3 organisms/ml of specimen. According to Toman 1979, cultural techniques may detect as few as 10-100 organisms/ml of sputum. Growing culture also permits the specific identification of AFB and determination of drug susceptibility.

3.6.3.4.1 Homogenization and decontamination

The majority of the clinical specimens submitted to the TB culture laboratory are contaminated to varying degree by more rapidly growing normal flora. These would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow. Most specimens must, therefore be subjected to a harsh digestion and decontamination procedure that liquefies the organic debris and eliminates the normal flora (WHO, 1998).

Theoretically, there are many different techniques available but none of them will selectively destroy only contaminating flora and achieve the complete liquefaction of the specimen. So, the main aim is to destroy as much of the contaminating flora as possible while harming as few mycobacteria as possible (Murray *et al.,* 1990).

Homogenization of clinical samples is essential to release the mycobacteria from the body fluid or tissue in which they are contained. Many different methods of homogenization and decontamination of sputum specimens for culturing have been described but there is no universally recognized best technique. One of the widely used methods in developing countries is Sodium hydroxide (Modified Petroff) method. It is used because of its relative simplicity and the fact that the reagents are easy to obtain. Other variety of more expensive and labor intensive homogenization and decontamination methods available are N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method, Zephiran-trisodium phosphate (Z-TSP) method, Oxalic acid method, Sulphuric acid method etc. The choice of a suitable method is to a large extent determined by the technical capability and the availability of staff in a laboratory, as well as the quality and type of equipments available (WHO, 1998).

3.6.3.4.2 Culture media for isolation of mycobacteria

Many different media are available for the isolation of mycobacteria. Tubercle bacilli are obligate aerobes and will not grow in the absence of oxygen, even a moderate reduction in the oxygen tension result in an appreciable decrease in the metabolism of the bacilli. The bacilli grow slowly, the generation time *in vitro* being 14-15 hours. Colonies appear only in about two weeks and sometimes may be delayed up to 6 to 8 weeks. Optimum temperature is 37°C and growth does not occur below 25°C or above 40°C. Optimum p^H is 6.4 to 7.0 and grows only in especially enriched media containing egg, asparagines, potatoes; serum and meat extract (Forbes *et al.,* 2002).

Solid media

Solid media, such as “Agar-based” media (Middlebrook medium) or “Egg-based media” (Lowenstein-Jensen medium), are recommended because of the development of characteristic, reproducible colonial morphology, good growth from small inocula, and a low rate of contamination (Forbes *et al.,* 2002). L-J and Ogawa media are the most widely used media; visible colonies appear after 2-6 weeks incubated at 37°C. Culture should be incubated for 6-8 weeks before being discarded. Colonies of tubercle bacilli on solid media typically appear as interwined cords that are heaped up and dry, and that may often have a smoother veil of growth surrounding the central formation. The eugonic colony (rough, tough and buff) form has often been described to have the appearance of bread crumbs and is tenacious and not easily emulsified.

Liquid media

Liquid media are not generally employed for routine cultivation but are used for sensitivity test, biochemical tests and preparation of antigens and vaccines. Mycobacteria grow as a surface pellicle in liquid medium unless a surfactant, such as the non-ionic detergent Tween 80, is added to lower the surface tension and permit diffuse growth. In general, the use of liquid media system reduces the turnaround time for isolation of acid-fast bacilli to approximately 10 days, compared with 17 days or longer for conventional solid media (Forbes *et al.,* 2002). Growth of mycobacteria in liquid media, regardless of the type, requires 5-10% CO_2 gas. Virulent strains often grow as twisted rope like colonies called as” serpentine cords”. Different types of liquid media are available for the isolation of mycobacteria like Herman Kirchner liquid medium, Dubos oleic acid-albumin liquid medium etc.

Biphasic media

Another media use for the culture of mycobacteria in some laboratories is the BBL-Septi-Chek AFB system, which is biphasic medium, containing both agar and broth. It

uses Middlebrook 7H9 broth in its lower chamber and agar slope of Middlebrook 7H11 in the upper part. Mycobacteria readily grow in this medium and at 2 or 3 days intervals during incubation the broth is made run over the agar. Colonies are farmed on the agar if they contain mycobacteria.

Liquid automated culture systems

The Bactec 460 TB instrument was the first automated instrument using radiometric detection system, developed by BACTEC. The MB/Bac T system is the first fully automated non-radiometric system for the culture of mycobacteria. BACTEC MGIT 960 uses tubes in which a fluorescent compound is embedded in silicone on the bottom of tubes. Growth of mycobacteria may be detected within 5-7 days, but positive results require further testing to distinguish between tubercle bacilli and other mycobacteria.

3.6.3.4.3 Inoculation and incubation

Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted. Either loops or pipettes can be used for primary cultivation, although plastic Pasteur pipettes are recommended (WHO, 1998). Each slope should be inoculated with 0.2-0.4 ml (2-4 drops or 2-4 loopful) of the centrifuged sediment, distributed over the surface. Two slopes are inoculated per specimen.

All cultures are incubated at 35-37°C until growth is observed or discarded as negative after eight weeks. Inoculated media should preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum, and then the media are further incubated with tight caps. The various Middlebrook agars require an atmosphere of 10% CO_2 and 90% air to ensure growth. CO_2 is not essential to initiate growth on egg-based media but does stimulate earlier and more luxuriant growth.

3.6.3.5 Identification tests

Identification of mycobacteria can be rather complex and needs a multitude tests ascertain to which species a mycobacterium belongs. Many different types of biochemical and other tests are described to identify mycobacteria.

3.6.3.5.1 Growth rate and growth at 25°C and 42°C

Tubercle bacilli grow slowly compared to other non-pathogenic acid-fast organisms, taking more than 7 days to appear on culture media. Similarly, *M. tuberculosis* and *M. bovis* do not grow at 25°C and 42°C. The optimum temperature for the growth of these mycobacteria is 37°C.

3.6.3.5.2 Growth on medium containing p-nitrobenzoic acid (PNB)

Para-nitrobenzoic acid has been used for the selective screening of *M. tuberculosis*. Human and bovine type of tubercle bacilli can be differentiated from all other mycobacteria in their inability to grow in L-J medium containing PNB. According to WHO (1998), in laboratories where facilities and reagents for niacin and nitrate testing are not available, identification of tubercle bacilli may be done by a combination of one or more of the catalase tests with growth at 25°C on L-J medium and growth on L-J medium containing PNB at 37°C. *M. tuberculosis* does not grow within three days at 37°C and does not grow at all at 25°C or on PNB medium. According to Fujiki (2001), *M. tuberculosis* does not grow on PNB at 4 weeks of incubation and even if few colonies are observed on the medium, it should be regarded as negative. All other mycobacteria are resistant to PNB.

3.6.3.5.3 Niacin test

Although, all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its diagnosis. Before the niacin

test is done, cultures should be checked for purity by microscopy; be 3-4 weeks old on egg based medium; and have sufficient growth of 50 or more colonies. The test may be done either with chemical reagents or with commercially available paper strips. Regardless of the method used, niacin is usually detected by its reaction with a cyanogens halide in the presence of the primary amine (Kent *et al.*, 1985).

3.6.3.5.4 Catalase test

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen, i.e. $2H_2O_2 \longrightarrow 2H_2O + O_2$. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually, all mycobacteria passes catalase enzyme, except for certain Isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis.* Mycobacteria posses several kinds of catalase that vary in heat stability: Drop catalase test (indicates the presence of catalase); Semiquantitative catalase test (indicates level of catalase production); and 68°C heat labile catalase test (indicates loss of catalase activity due to heat). If niacin test is not available, 68°C labile catalase test can be used to differentiate *M. tuberculosis* from other mycobacteria (Fujiki, 2001).

3.6.3.5.5 Nitrate test

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allow this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria. This test is based on the principle that the enzyme nitrate reductase causes the reduction of nitrate in the presence of a suitable electron donor to nitrite or nitrogen. *M. tuberculosis* produces varying degree of coloration during the test, which are influenced by the age of the culture, temperature, enzyme inhibitors, and hydrogen ion concentration.

3.6.3.5.6 Urease test

The ability of a culture to hydrolyze urea releasing ammonia ($NH_2CONH_2 + H_2O$ gives $2NH_3 + CO_2$) is useful in identifying both scotochromogens and nonphotochromogens.

M. scrofulaceum, M. flavescens, M. bovis, M. tuberculosis, and *M. gastri* are positive, whereas *M. avium* complex, *M. xenopi*, *M. terrae* complex, and *M. gordonaei* are negative.

3.6.3.5.7 Pyrazinamidase test

The deamidation of PZA to pyrazinoic acid and ammonia is helpful in separating *M. marinum* (positive in 4 days) from *M. kansasii* (negative), and weakly niacin positive strains of *M. bovis*(negative even at 7 days) from *M. tuberculosis* (positive within 4 days) and the *M. avium* complex.

3.6.3.6 Serological tests

The availability of monoclonal antibodies to, and cloned or synthetic antigens of, *M. tuberculosis* offers new prospects for the development of a specific serological test but no clinically acceptable test has emerged owing to the unacceptably high incidence of misleading results (Grange, 1998).

3.6.3.6.1 Enzyme linked immunosorbent assay (ELISA)

Application of the ELISA procedure to the sera of patients with active TB has shown elevated, specific titers in the IgG fraction when PPD-S is used as an antigen. The demonstration of an elevated IgG antibody against *M. tuberculosis* could be very useful for the identification of patients with active TB whose sputum smears and cultures are negative (Inderlied *et al.,* 2003).

3.6.3.6.2 MycoDot antibody test

Recently, a new simple to perform, 20 minutes immunoassay, the mycodot antibody test has become available. It is a comb dipstick, manufactured by Mossman associates. Mycodot uses purified lipoarabinomannans (LAMs) as antigen (highly immunogenic lipopolysaccharide found in the cell wall of all mycobacteria). Mycodot has been

designed for diagnostic use, i.e. it detects anti-mycobacterial antibody levels likely to be found in those with active disease, i.e. high levels. A mycodot test should therefore only be preformed on those with suspected Tb (it is not a screening test). A positive test indicates active TB. A negative mycodot test, however, cannot exclude TB because a negative result may be due to a low antibody titer as occurs in immunosuppression, all circulating antigen-antibody complexes lowering the antibody titer, or to extra-pulmonary TB in which the antibody level is below that which can be detected by the test (Cheesbrough, 2002).

3.6.3.7 Tuberculin test

The tuberculin test is the only means of estimating the prevalence of infection in a population (Park, 2005). There are three main tests: the mantoux intradermal test, the heaf test, and the time multiple puncture test. The mantoux test in the most satisfactory of all tests for epidemiological studies, as an exact amount of material can be introduced into the skin and the degree of reactivity can be accurately assessed by measuring the diameter of indurations (Grange, 1998).

Tuberculin is a purified protein derived from tubercle bacilli. Another name for tuberculin is PPD (Purified protein derivative). Following infection with *M. tuberculosis*, a person develops hypersensitivity to tuberculin. Tuberculin injected into the skin of an infected person produces a delayed local reaction after 24-48 hours. This reaction is quantified by measuring the diameter of skin induration (thickening) at the site of the reactions. The reaction indicates hypersensitivity. In other words, the reaction only shows that the person has at sometime be infected with *M. tuberculosis* (WHO, 2004). Various conditions can also suppress this reaction. So, weak or negative tuberculin reaction does not exclude active TB. Some persons are intrinsically non-reactive and in others cell-mediated immune-reactivity is depressed due to advanced age, malnutrition, other concurrent diseases, or advanced TB (Grange, 1998).

3.6.3.8 New techniques for diagnosing tuberculosis

Most of the recent advances in the laboratory diagnosis of TB have been directed at the development of rapid culture, identification, and drug susceptibility systems for use in TB specialist laboratories (Cheesbrough, 2002). Some advanced techniques that can be used for the diagnosis of TB include: Bactec 460 TB rapid radiometric culture system, Bactec 9000 MB system, Septi-check AFB system, Mycobacteria growth indicator tube (MGIT), ESP culture system II, Polymerase chain reaction (PCR), Bacteriophage-based test to detect *M. tuberculosis* in sputum, chromatographic analysis etc. Thus, several new technologies have been introduced that are promising in terms of being faster and more reliable.

3.7 Antimicrobial susceptibility testing (AST)

Susceptibility testing is used to determine the resistance of strains isolated before the commencement of treatment (initial or primary resistance); or to discover whether resistance has arisen during treatment (secondary resistance). For most newly diagnosed patients with TB they will be treated with two or more of the primary anti-tubercular drugs. But if the patient has previously received anti-tubercular drugs, it is important to determine the susceptibility pattern, as it is in patients with recurrent TB that drug resistance is most commonly seen (Inderlied *et al.,* 2003).

Susceptibility test is done to find out whether the organisms isolated in a culture is susceptible or resistant to anti-tubercular drugs used for the treatment of a patient. Development of primary drug resistance in TB represents an increase in the proportion of resistant organisms. This increase in resistant organisms results from a spontaneous mutation and subsequent selection to predominance of these drug-resistant mutants by the action of a single, ineffective or inappropriate drug therapy. And therapy directed against *M. tuberculosis* is dependent on the susceptibility of the isolate to various antimicrobial agents. So, AST is very important and should be performed appropriately. It informs an important aspect of TB control. Susceptibility testing of *M. tuberculosis*

requires meticulous care in the preparation of the medium, selection of adequate samples of colonies, standardization of the inoculum, use of appropriate controls, and interpretation of results (Forbes *et al.,* 2002).

3.7.1 Direct vs. indirect susceptibility testing

Susceptibilities may be performed by either the direct or the indirect methods. The direct method uses as the inoculum a smear positive concentrate containing more than 50 AFB per 100 oil immersion fields; the indirect method uses a culture as the inoculum source. Although, the direct testing provides earlier rapid results, the results may be unsatisfactory because of contamination or low numbers of colony-forming units. So, this method is less standardized (Forbes *et al.,* 2002).

3.7.2 Conventional methods

Different conventional methods used for determination of antimicrobial susceptibility pattern are the resistance ratio, the proportion, the absolute concentration and the disc diffusion methods. These generally accepted methods for the determination of the drug susceptibility of mycobacteria are based on the growth of the organism on a solid medium. Drug resistance is defined for MTC in terms of the critical concentration of the drug. The critical concentration of a drug is the amount of the drug required to prevent growth above the 1% threshold of the test population of tubercle bacilli (Forbes *et al.,* 2002).

3.7.2.1 Resistance ratio method (RR method)

The RR method is performed either on solid media (egg or Middlebrook agar) or in liquid media (Middlebrook or Dubos broth). This method compares the resistance of unknown strains of tubercle bacilli (test organism) with that of a standard laboratory strain $H_{37}Rv$, or preferably three wild strains, taking their modal resistance as control. Parallel sets of media containing two-fold dilutions of the drug are inoculated with the standard inoculum prepared from both the unknown and the standard strain of tubercle

bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test strain to the MIC of the standard strain in the same test (Murray *et al.*, 1990).

3.7.2.2 Proportion method (PR method)

It is a quantitative test, in which several dilutions of standardized inoculum are inoculated onto control (drug free) and drug containing agar medium. The decrease of growth in drug-containing media and drug-free media are compared. If growth at the critical concentration of a drug is more than 1%, the isolate is considered clinically resistant (Forbes *et al.*, 2002). It cannot be performed in liquid media because it is a quantitative test, except in the case of the Bactec, where quantification of growth is possible (Collee *et al.*, 1996).

Both the RR and the PR methods are expensive and time consuming and require rigorous quality control if reliable results are to be obtained (Grange, 1998).

3.7.2.3 Absolute concentration method

This method is used in the USA and parts of Europe to determine test strain MICs. The tests are usually performed on Middlebrook's 7H10 medium. As the concentration of drug in the media is critical, media that are heated after addition of the drug, such as egg media, are unsuitable (Collee *et al.*, 1996). For each drug tested, a standardized inoculum is inoculated to control (drug-free) media and media containing several appropriate graded drug concentrations. Resistance is expressed as the lowest concentration of drug that inhibits all or almost all of the growth, that is, the MIC (Forbes *et al.*, 2002).

3.7.2.4 Disc diffusion method

This method is possible if the mycobacterium is a "rapid grower". Agar plates are flood-seeded with the test organism, antibiotic discs are applied and the plate is then placed in

a shield bag and incubated for appropriate time and temperature. The zone size is then recorded and the results reported (Collee *et al.,* 1996). So, this method which rely on the size of zone of inhibition surrounding a drug containing disc, are not suitable for the slowly growing mycobacteria because the drug diffuses out of the disc throughout the medium before the organism has a chance to grow (Inderlied *et al.,* 2003).

3.7.3 Bactec radiometric method

Bactec radiometric methods are most commonly is used in the US which require Bactec 460 TB instrument. Employing the principles of PR method, this rapid method uses liquid medium containing ^{14}C-labelled growth substrate. Growth is indicated by the amount of ^{14}C-labelled-CO_2 released, as measured by the Bactec 460 instrument and converted proportionally to a quantitative growth index (GI) on a scale of 0 -999. For each drug tested, a standardized inoculum is inoculated into a drug free and drug containing vials. The rate and amount of CO_2 produced in the absence or presence of drug is then compared (Forbes *et al.,* 2002). This method has allowed susceptibility test results to be produced in 1-2 weeks which has greatly reduced the time taken for susceptibility testing.

3.7.4 New approaches

Several new technologies recently have been introduced that are promising in terms of being faster, more reliable, and/or easier to perform than most conventional methods for susceptibility testing. For example, the MGIT system has been used to perform *in vitro* susceptibility testing of *M. tuberculosis,* and mutations leading to RFP resistance have been detected using molecular methods. One molecular method, the line probe assay, is a commercially available revere hybridization-based probe assay for rapid detection of RFP mutations leading to RFP resistance in *M. tuberculosis.*

High-density DNA probe assays, luciferase-reporter mycobacteriophage assays are some of the molecular approaches applied to susceptibility testing of *M. tuberculosis.* In addition to these, the E-test has been successfully applied (Forbes *et al.*, 2002).

3.7.5 Possible consequences of inaccurate drug susceptibility testing

The consequences of inaccurate susceptibility testing may be as follows:

- Misclassification of strains
- Unnecessary changes of chemotherapy
- Use of reserve drugs, leading to:
    - More toxicity
    - Less chance of cure
    - More difficult management
    - The need for hospitalization
    - More laboratory work
- More staff needed
- Higher cost

Resistance strains may be misclassified as sensitive, and vice versa. If sensitive strains are reported as resistant, regimens may be changed unnecessarily and reserve drugs; if available, may be introduced. However, such drugs are usually more toxic, less effective and more costly than the drugs used for primary chemotherapy.

The management of patients reserve drugs while ambulatory may become too difficult. Such patients often have to be hospitalized for a long time, which costs about twelve time as much as domiciliary treatment. More staff will be needed, in particular for the additional laboratory work required (repeated tests of kidney and liver function, blood examinations and close bacteriological follow-up) and this will add to the cost of hospital treatment. Thus there may be heavy drain on resources allocated to therapeutic services, merely as a consequence of inaccurate susceptibility tests. So, the use of

appropriate drug susceptibility test is one of the most effective ways of avoiding the development of drug resistance-a man-made problem (Toman, 1979).

3.8 Treatment

TB is a curable disease. Drug treatment is the only effective method to control active disease of TB. In order to prevent the emergence of drug resistant mutants, which are present initially in very small numbers, at least two effective drugs are always required. The total duration of treatment may be 6-9 months. Because of the long generation time of mycobacteria and their long periods of metabolic inactivity, prolonged courses of drug therapy are always necessary.

Five drugs regard as essential in the treatment of TB: Isoniazid (INH), Rifampicin (RFP), Pyrazinamide (Z/PZA), Streptomycin (SM) and Ethambutol (EMB). Thioacetazone (T) is also used to supplement INH in many developing countries because of its low cost (WHO, 1993). Three main properties of anti-TB drugs are: bactericidal ability, sterilizing ability and the ability to prevent resistance. The anti-TB drugs posses these properties to different extents. INH and RFP, the most powerful bactericidal drugs, active against all populations of TB bacilli. PZA and SM are also bactericidal against certain populations of TB bacilli. PZA is active in an acid environment against TB bacilli inside macrophages. SM active against rapidly multiplying extra cellular TB bacilli. EMB and T are bacteriostatic drugs, used in association with more powerful bactericidal drugs to prevent the emergence of resistant (WHO, 2003).

DOTS (Directly observed treatment short course), a TB control strategy, pioneered by international union against TB and lung disease (IUATLD) and recommended by WHO to ensure cure by providing the most effective medicine and confirming that it documented to be effective worldwide on a programme basis. This strategy controls TB by giving drugs to patients under direct observation of health workers. This means that health workers support the patients during the course of treatment and watch the patient

swallow the tablets. DOTS has been found 100% effective to cure TB and to prevent MDR. Only DOTS ensure cure of diagnosed TB patients. It can also prevent relapse and death. DOTS introduced in Nepal in 1996, and successfully implemented throughout the country since April 2001. By July 2006 DOTS had been expanded to 560 treatment centers with 2795 sub-centers. The treatment success rate in DOTS is now 88% in Nepal (NTC, 2007).

3.8.1 Treatment regimens

The chemotherapeutic regimens are based on standardized combinations of 5 essential anti-TB drugs viz: INH, RFP, SM, EMB and PZA/Z to prevent the emergence of resistant strains. Each standardized chemotherapeutic regimens consists of 2 phases:

- Initial (intensive) - 2 to 3 months, with 3-5 drugs given daily under direct observation, to maximally reduce the number of TB organism. The number of drugs used relates to the risk of failure of treatment due to bacterial resistance; &
- Continuation – 4 to 6 months, with 2-3 drugs given 3 times a week under direct observation, or in some cases (e.g. during repatriation of refugee) 2 drugs for 6 months given daily unsupervised, but in fixed-dose combination form.

Treatment categories are essential for prioritization of TB treatment according to public health risk-category I is the highest priority, category III the lowest (WHO 1997).

Category I

These patients are:

- Smear-positive persons who have never previously been treated or have only received treatment for less than one month.
- Severely ill patients with other forms of TB (new smear-negative PTB with extensive parenchymal involvement, and new cases of severe forms of TB).

Recommended regimens

Intensive phase: 2HRZE (S) i.e. Isoniazid, Rifampicin, Pyrazinamide and either Ethambutol or Streptomycin, daily for 2 months.

Continuation phase: 4HR or $4H_3R_3$ i.e. Isoniazid and Rifampicin given daily or three times a week for 4 months. For patients with TB meningitides, disseminated TB or Spinal disease with neurological complications, Isoniazid and Rifampicin should be given daily for 6-7 months (i.e. a total of 8 months of therapy).

Category II

Patients who were previously treated and now sputum smear-positive, include:

- Treatment after interruption.
- Treatment failure and
- Relapse after treatment

Recommended regimens

Intensive phase: 2HRZES/ 1HRZE i.e. Rifampicin combined with Isoniazid, Pyrazinamide and Ethambutol, given daily for 3 months and supplemented with Streptomycin for the first 2 months.

Continuation phase: $5H_3R_3E_3$ i.e. Isoniazid, Rifampicin and Ethambutol three times per week for 5 months.

Category III

These patients are:

- Smear-negative pulmonary patients (with limited parenchymal involvement),
- Non-serious extra pulmonary disease in adults and children (including symptomatic primary disease)

Recommended regimens

Intensive phase: 2HRZ/ $2H_3R_3Z_3$ i.e. Isoniazid, Rifampicin and Pyrazinamide given daily or three times a week for 2 months.

Continuation phase: 6HE (T)/ $4H_3R_3$ i.e. Isoniazid and Ethambutol or Thioacetazone, given daily for 6 months, or Isoniazid and Rifampicin three times a week for 4 months.

Table 2. Recommended treatment regimens for each treatment category (WHO, 1997)

Treatment Category	Patients	Initial (Intensive) Phase	Continuation Phase
I	New smear-positive PTB; new smear –negative PTB with extensive parenchymal involvement; new cases of severe forms of extra-pulmonary TB.	2 EHRZ (2 SHRZ) Or $2 E_3H_3R_3Z_3$ $(2 S_3H_3R_3Z_3)$	$4 H_3R_3$ (6 HE)
II	Sputum-smear positive; relapse; treatment failure; treatment after interruption.	2 HRZE/ 1 HRZE	$5 H_3R_3E_3$
III	New smear-negative PTB (other than in Category I); new less severe forms of extra-pulmonary TB.	2 HRZ Or $2 H_3R_3Z_3$	$4 H_3R_3$ (6 HE)

Modern anti-tubercular drug cures most tuberculosis case if an effective combination of drugs, in proper doses used for a sufficient period of time. However in practice, many cases are not cured (Malla, 1996). Drug resistant tuberculosis is a case of tuberculosis (usually pulmonary) excreting bacilli resistant to one or more anti-tuberculosis drugs. Multi-drug resistant (MDR) tuberculosis is resistant to at least Isoniazid and Rifampicin, the main anti-tuberculosis drugs. The emergence and spread of MDR TB threat the global TB control.

Above mentioned five antimicrobial agents are referred to as primary drugs. If any drug resistance is detected to any of the primary drugs, a second battery of agents is tested.

These secondary drugs include ethionamide, capreomycin, ciprofloxacin, ofloxacin, kanamycin, cycloserine and rifabutin, which are more toxic.

3.8.2 Adverse effects of anti-TB drugs

Adverse effects are classified as minor or major. If a patient develops a major side effect, the treatment or the offending drug is stopped. Patients with major adverse reactions should be managed in a hospital (WHO, 1993; WHO, 1997).

Table 3. Symptoms based approach to adverse effects of anti-TB drugs

Side effects	Drug (s) probably responsible	Management
Minor		
Anorexia, nausea, abdominal pain	Rifampicin	Continue anti-TB drugs, check drug doses. Give drugs last thing at night.
Joint pains	Pyrazinamide	Aspirin
Burning sensation in feet	Isoniazid	Pyridoxine 100 mg daily
Orange/ red urine	Rifampicin	Reassurance
Major		
Itching of skin, skin rash	Thioacetazone (Streptomycin)	Stop anti-TB drugs
Deafness	Streptomycin	Stop Streptomycin, use Ethambutol
Dizziness	Streptomycin	Stop Streptomycin, use Ethambutol
Jaundice	Most anti-TB drugs (especially Isoniazid, Pyrazinamide and Rifampicin)	Stop anti-TB drugs
Vomiting and confusion (suspect drug-induced acute liver failure)	Most anti-TB drugs	Stop anti-TB drugs, urgent liver function tests and prothrombin time.
Visual impairment	Ethambutol	Stop Ethambutol
Shock, purpura, renal failure	Rifampicin	Stop Rifampicin

3.9 Drug resistant tuberculosis

Drug resistance by definition, temporary/permanent capacity of the organisms and their progeny to remain viable or to multiply in presence of concentration of the drug that would normally destroy/inhibit the growth of other similar cells. So, drug resistant TB is a case of TB (usually PTB) excreting bacilli resistant to one or more anti-tubercular drugs. Resistance of *M. tuberculosis* to anti-TB drugs is the result of a spontaneous genetic event and, worse "a man-made amplification of the natural phenomenon" (Pfyffer, 2000).

3.9.1 Types of drug resistance

3.9.1.1 Primary or pre-treatment resistance

It occurs in patients who have not had prior treatment with anti-tubercular drugs. This type of resistance is observed when a patient develops TB after being infected by another patient who has resistant TB organisms.

3.9.1.2 Acquired or secondary resistance

It occurs in patient with some record of previous drug treatment (WHO, 2000). Acquired drug resistance was defined as resistance in cultured isolates from patients who gave a history of previous drug treatment for longer than one month or the bacteria were sensitive to the drug at the start of the treatment but became resistant to the particular drug during the course of treatment with it (Park, 2005). This is mostly because of single drug-due to irregular drug supply inappropriate prescriptions or poor adherence to treatment suppresses the growth of the susceptible bacilli to that drug but permits the multiplication of drug resistance organisms (Rijal *et al.*, 2002).

3.9.2 Mechanism of resistance

Resistance to anti-tuberculosis medications is due to unlinked chromosomal mutations of the tubercle bacilli. In *M. tuberculosis* spontaneous mutations occur at a frequency of approximately 10^{-5} to 10^{-8} (Pfyffer, 2000; Iseman, 1993). Since resistance to various drugs arises independently, the likelihood of spontaneous mutation to Isoniazid and Rifampicin, for instance, is 1 in 10^{14} ($10^{6} \times 10^{8}$). At first sight, the probability of dual mutation seems minimal. However, since PTB is always associated with enormous bacterial masses (cavities contain as many as 10^{7}-10^{9} organisms), dual mutations will be seen with a certain frequency. This threat of multi-drug resistance is one reason why combination regimens must always used for TB.

Resistance to RFP arises due to mutations in the beta subunit of RNA polymerase encoded by rpo B, inhibiting RNA synthesis. Missense mutation in RNA polymerase and alteration in cell wall permeability is responsible for RFP resistance. Similarly, resistance to INH could occur either through a miss sense mutation that blocks INH from binding to the gene or through formation of large amounts of enzyme which could nullify the effect of INH. Kat G gene product has also been shown to be responsible for INH resistance. Resistance to streptomycin emerges through mutations in rrs and rpsl gene that produce alteration in the streptomycin binding site. Most pyrazinamide resistant organisms have mutations in the pyrazinamidase gene, although the gene may also be inactivated through the insertion of IS6110 (Gillespie, 2002).

3.9.3 Multi-drug resistant TB (MDR-TB)

MDR-TB refers to *M. tuberculosis* isolates that are resistant to at least both INH and RFP, the two most powerful anti-TB drugs. The emergence and spread of MDR-TB threat global TB control. People with MDR-TB disease can only be treated with reserve or second line drugs. These drugs are not as effective as the first line drugs and cause more side effects. The susceptibility patterns of *M. tuberculosis* isolates against anti-tubercular drugs informs an important aspect of TB control, and surveillance and

analysis of local rates of TB-drug resistance helps in the detection and monitoring of the extent of MDR strains, indicating the quality of TB control in the country.

People who have spent time with someone sick with MDR TB disease can become infected with TB bacteria that are resistant to several drugs. Historically, MDR TB has spread gradually with alarming rates seen in 2001 in India, China, UK, Russia, Peru, Spain and Puerto Rico (WHO, 2004; Swaminathan *et al.*, 2005). The magnitude of anti-TB drug resistance is not well documented in the SEAR; however, the mean prevalence of MDR TB among new smear positive cases in the SEAR is believed to be low, at an overall 2%. In 1996/97, an MDR TB case was 1.20%, with 3.60% in 1998/99 which later decreased to 1.30% in 2001/2002 (NTC, 2007). Isolated reports of higher levels of MDR TB are reported mainly from hospital settings. Levels as high as 60% are reported among previously treated cases in tertiary care facilities.

3.9.4 Extensive drug-resistant TB (XDR-TB)

XDR-TB or extensive drug-resistant TB, is currently defined as resistance to the two most potent anti-TB drugs-INH and RFP, and resistance to at least two of the six classes of second-line drugs i.e. aminoglycosides, polypeptides, fluoroqunolones, thioamides, cycloserine and para-aminosalicyclic acid. These strains leave patients without treatment options that meet international standards and are therefore virtually untreatable (WHO, 2007).

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

The media, reagents and chemicals used in this research work were procured from renowned manufacturers. The detail is attached in Appendix II. Sputum samples were obtained from patients visiting NTC, who gave verbal consent to participate in the study.

4.2 Methods

4.2.1 Study design

This study was a cross-sectional study undertaken to diagnose the suspected TB patients, to isolate and identify *M. tuberculosis* and compare drug susceptibility pattern of the isolates using proportion and resistance ratio methods.

4.2.2 Study setting

This study was a collaborative study of Central Department of Microbiology, Tribhuvan University, Kirtipur with National Tuberculosis Centre, Thimi, Bhaktapur.

4.2.3 Study population

All the suspected tuberculosis patients attending NTC, Thimi, Bhaktapur were enrolled for the study.

4.2.4 Study period

The study was carried out from September 2006 to June 2007.

4.2.5 Sample size

A total of 862 persons visiting NTC, Thimi, Bhaktapur were included in this study. The patients were examined for acid fast bacilli (AFB) by fluorescence microscopy. Smear positive samples and only those smear negative samples requested fro culture were further processed for the study. For all culture positive samples, drug susceptibility testing were performed by both proportion and resistance ratio methods.

4.2.6 Data collection and analysis

Standard questionnaires were prepared and used to collect the informations of each patient on personal informations (age, sex, case type etc.) (Appendix I). Data was analyzed using SPSS version 11.5 system (Statistical package for Social Sciences).

4.3 Laboratory methodology

4.3.1 Collection of sputum sample

Sputum samples were collected following standard protocol (WHO, 1998). Briefly as:

- Day 1
  Sample 1- suspect provided an “on the spot” sample.
- Day 2
  Sample 2- suspect brings an early morning sputum sample.
- Day 3
  Sample 3- suspect provided another “on the spot” sample.

The sputum was collected in a wide mouthed, transparent, plastic, sterile, leak-proof, screw capped container. About 4 ml mucopurulent sputum was collected.

4.3.2 Sputum evaluation

When a sputum specimen was being collected, adequate safety precautions were taken to prevent the spread of infectious organisms. The container was labeled and filled in a

request form available in the hospital. The request form contained patient's full name, date, and address and lab number. The specimen was processed within two hours or if not processed kept at 4°C. The collected specimen was first stained by fluorochrome method and then cultured.

4.3.3 Macroscopic examination of sputum

Macroscopic examination included the observation of the colour and appearance of sputum sample (Cheesbrough, 2002). The sputum was described as follows:

Table 4. Macroscopic examination of sputum sample

S No.	Type	Observation
1	Purulent	Green looking, mostly pus
2	Mucopurulent	Green looking with pus and mucus
3	Mucoid	Mostly mucus
4	Mucosalivary	Mucus with a small amount of saliva
5	Blood stained	Presence of blood

When the sputum was mostly saliva, the specimen was reported as "unsuitable" for microbiological investigation and requested another specimen.

4.3.4 Microscopic examination of sputum

4.3.4.1 Sputum smear preparation

A small proportion of purulent and mucopurulent or bloody material was selected, separated from the remainder with a wooden stick; smear was prepared and stained as per standard protocol (WHO, 1998). Detailed guideline for the sputum smear preparation is mentioned in Appendix IV.

4.3.4.2 Direct smear microscopy by fluorochrome method

1. The heat fixed slides were placed on a staining rack in batches (maximum 12), with smears facing up.
2. The Auramine solution was poured on the slide to cover the entire smear and followed to stand for 15 minutes.
3. After 15 minutes the slides were rinsed with distilled water and drained.
4. The slides were then decolorized with 20% H_2SO_4 for 2-3 minutes.
5. After 2-3 minutes the slides were rinsed with distilled water and drained.
6. The slides were then flooded with Methylene blue, counter stain for 10-15 seconds.
7. The slides were gently rinsed with distilled water and drained.
8. Then after, the slides were dried and the whole smear was examined in fluorescence microscope.

With fluorochrome staining, tubercle bacilli are rod shaped and emit a bright yellow fluorescence against a dark background.

Table 5. Interpretation of stained smear by fluorescence microscopy (WHO, 1998)

Range	Report	Fluorescence microscopy magnification		
		250X	450X	630X
0	No AFB seen	0	0	0
1-9/100 fields	Report exact count	Divide observed count by 10	Divide observed count by 4	Divide observed count by 2
10-99/100 fields	+1			
1-10/ field	+2			
>10/ field	+3			

The preparation and composition of staining reagents for fluorescence microscopy is mentioned in Appendix III.

4.3.5 Culture of sputum

The most reliable way of establishing diagnosis of any infectious disease is to isolate and identify the causative organisms in culture. The clinical specimens submitted for culture were sputum. They were cultured on primary culture medium- 2% modified Ogawa medium.

The composition and preparation of media for culture is mentioned in Appendix V.

4.3.5.1 Digestion and decontamination of sputum for culture

Specimen is subjected to digestion and decontamination procedure to free the bacilli from mucus, cells or tissue in which they may be embedded and to eliminate the unwanted normal flora. Sodium hydroxide (Modified Petroff) method was used for the digestion and decontamination of sputum.

The composition and preparation of media for culture is mentioned in Appendix IV.

Detailed procedure for NaOH (Modified Petroff) method as given by Fujiki (2001) is mentioned in Appendix IV.

4.3.5.2 Culture of digested and decontaminated sample

One drop of sediment was inoculated on each of two culture tubes of 2% modified Ogawa medium with the help of sterile Pasteur pipette. The caps of inoculated media were kept loose and incubated at 37°C lading the tubes on the slanting bed to ensure even distribution of inoculum. After 24 hours, the tubes were placed upright. When the surface of media became dry, the caps were tighten and further incubated at 37°C until growth was observed or discarded as negative after 8 weeks. A tube was also incubated at 25°C for upto 8 weeks.

4.3.5.3 Culture examination

All the cultured tubes were examined after one week to detect rapidly growing mycobacteria which may be mistaken for *M. tuberculosis*. The growth on the media was observed at every week after inoculation. The negative cultures were discarded only after eight weeks.

4.3.5.3.1 Recording and reporting of culture results

Since culture report should be qualitative and quantitative, it was reported as shown in table six as recommended by WHO (1998):

Table 6. Recording and reporting of culture

S No.	Reading	Report
1	No growth	Negative
2	1-19 colonies	Positive (number of colonies)
3	20-100 colonies	Positive (1+)
4	100-200 colonies	Positive (2+)
5	200-500 colonies (almost confluent growth)	Positive (3+)
6	> 500 colonies (confluent growth)	Positive (4+)
7	Contaminated	Contaminated

4.3.5.3.2 Microscopic examination of ZN staining

The acid-fastness of the growth was confirmed by ZN-staining. A very small amount of growth was removed from the culture using a loop and gently rubbed into one drop of sterile saline on a clean slide. The smear was allowed to dry, fixed by heat and stained by ZN method (Fujiki, 2001). The composition and preparation of staining reagents for ZN-microscopy is mentioned in Appendix III. The ZN staining procedure is described in detail in Appendix IV.

4.3.5.3.3 Subculture from Ogawa medium on LJ medium

The growth on Ogawa medium was sub-cultured on LJ medium for the identification of isolates by different biochemical tests and antimicrobial susceptibility test.

4.3.6 Identification of isolates

Preliminary identification of tubercle bacilli was made by colony morphology, non-pigmented colony, slow growers, do not emulsify in saline used for making smears but give a granular suspension and microscopically they are frequently arranged in serpentine cords of varying length and their inability to grow at 25°C. Besides it is best to do confirmatory tests which were carried out by various methods.

4.3.6.1 Biochemical tests

The colonies on the LJ media were further confirmed by conventional biochemical tests (Niacin and Catalase tests) as described by WHO (1998) and Fujiki (2001).

4.3.6.1.1 Niacin production test

1. 1 ml of sterile water was added to the culture slant. If the growth was confluent, the medium was punctured with the Pasteur pipette to allow contact of water with the medium.
2. The tube was placed horizontally so that the fluid covered the entire surface of the medium.
3. It was left for 30 minutes for the extraction of niacin.
4. The slants were raised upright for 5 minutes to allow the fluid to drain to the bottom.
5. 0.5 ml of the fluid extract was taken to a clean screw capped tube.
6. Sequentially, 0.5 ml of 4% aniline solution and 0.5 ml of 10% Cyanogen bromide solution was added.

7. The tubes were closed and the solutions were observed for the formation of yellow colour within 5 minutes.
    - Results and interpretations:
        - Negative: No colour change
        - Positive: Yellow colour appearing within 5 minutes. The colour appears as a ring at the interface of the two reagents, or if the tube is shaken, as a yellow column of liquid.
8. $H_{37}Rv$ strain was used as appositive control and testing the extract from un-inoculated tube of medium was used as negative control.
9. Cyanogen bromide is toxic. So, after performing the test, 2-3 ml of 4% NaOH was added to the tube for the detoxification and then discarded by autoclaving.

The composition and preparation of reagents for niacin test is given in Appendix VI.

4.3.6.1.2 Drop catalase test

1. The LJ medium having confluent growth was taken.
2. One to two drops of freshly prepared tween-peroxide mixture was added to the slant.
3. It was observed for the period of 5 minutes for the formation of gas bubbles which indicated positive test.

4.3.6.1.3 68°C labile catalase test

1. A bacterial suspension was made by suspending several loopful of growth into the screw-capped tube containing 0.5 ml of 0.067 M phosphate buffer solution.
2. The suspension was then incubated in 68°C water bath for 20 minutes.
3. Then the suspension was cooled to room temperature.
4. 0.5 ml of freshly prepared tween-peroxide mixture was added to the tube and recapped loosely.

5. The tube was observed without shaking for the bubble formation. Negative tubes were discarded only after 20 minutes.

The composition and preparation of biochemical reagents is given in Appendix VI.

4.3.7 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is an *in vitro* method for estimating the activity of drugs against an infecting microorganism *in vivo*. AST of all the isolates were performed by both proportion method and resistance ratio method. Each strain was tested against four primary anti-tubercular drugs by both methods. The drug concentrations used for proportion method were: INH- 0.25 µg/ml and 8.0 µg/ml; RFP- 20.0 µg/ml and 40.0 µg/ml; SM- 4.0 µg/ml and 8.0 µg/ml; and EMB- 1.0 µg/ml and 2.0 µg/ml. While for resistance ratio method, the concentrations were 0.5 µg/ml and 1.0 µg/ml; RFP- 32.0 µg/ml and 64.0 µg/ml; SM- 16.0 µg/ml and 32.0 µg/ml; and EMB- 4.0 µg/ml and 8.0 µg/ml.

4.3.7.1 Proportion method

4.3.7.1.1 Preparation of drug containing media

The drug containing media were prepared with different concentrations of primary anti-tubercular drugs as shown in Table seven.

Table 7. Concentrations of drugs used for proportion method

S No	Concentrations of drugs used	0.25 µg/ml	8.0 µg/ml	20.0 µg/ml	40.0 µg/ml	4.0 µg/ml	1.0 µg/ml	2.0 µg/ml
1	INH	√	√					
2	RFP			√	√			
3	SM		√			√		
4	EMB						√	√

The preparation of drug solutions and drug containing media the Antimicrobial susceptibility testing by proportion method is mentioned in Appendix VII.

4.3.7.1.2 Preparation of bacillary suspension

1. 0.1 ml of sterile distilled water was taken in a screw-capped homogenizer, containing seven 3 mm diameter plastic beads.
2. One loopful of growth from sub-cultured L-J medium was transferred to the tube by using standard 3 mm diameter loop.
3. The tube was vortexed for few minutes to give smooth suspension.
4. The density of the suspension was adjusted by comparing with a Mc Farland No. 1.
5. This gave 1 mg/ml bacillary suspension. This was the “neat suspension”.

The preparation of Mc Farland No. 1 is mentioned in Appendix V.

4.3.7.1.3 Dilution of bacillary suspension for inoculation

1. Serial dilution was made with the neat suspension by transferring 0.1 ml of neat suspension to tube containing 0.9 ml sterile distilled water and mixed well. This was 10^{-1} dilution (Suspension-1).
2. 0.1 ml of suspension-1 was transferred to tube containing 0.9 ml of sterile distilled water and mixed well. This was 10^{-2} dilution (Suspension-2).
3. Again, 0.1 ml of suspension-2 was transferred to tube containing 0.9 ml of sterile distilled water and mixed well. This was 10^{-3} dilution (Suspension-3).

4.3.7.1.4 Inoculation and incubation

1. From 10^{-1} dilution, eight drug containing media of different concentrations as shown in table seven were inoculated with one loopful of bacillary suspension.

2. Similarly from each dilution, three controls of plain L-J media were inoculated with the respective bacillary suspensions i.e. 10^{-1} dilution in control I, 10^{-2} dilution in control II and 10^{-3} dilution in control III.
3. All these tubes were incubated at 37^0C for 4 weeks and observed at weekly intervals.

4.3.7.1.5 Interpretation of results

10^{-3} dilution represents 1% of 10^{-1} dilution. Therefore, any colonies growing on drug containing medium inoculated with the 10^{-1} dilution that equal or more the number of colonies growing on the control medium inoculated with the 10^{-3} dilution (Control III) represents 1% or more of the test population.

The growth density (if possible no. of colonies) on the culture media with critical concentration of the corresponding drug was compared with the growth on Control III.

Table 8. Critical concentrations of primary anti-tubercular drugs used in the study

S. No.	Drugs used in the study	Concentrations (µg/ml)
1	INH	0.25
2	RFP	40.0
3	SM	4.0
4	EMB	2.0

Sensitive (S)

When there was no growth on the culture media with the “critical” concentration of the respective drugs, then a strain of TB bacteria was classified as “sensitive”.

Resistant (R)

When the growth on the culture media with the "critical" concentration of the respective drug was equal or more than on control III, a strain of TB bacteria was termed "resistant".

The result was considered questionable, if the growth on the culture media with the "critical" concentration of the respective drug was less than the growth on control III. The questionable test results were repeated from the beginning.

4.3.7.2 Resistance ratio method

This method compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain $H_{37}Rv$.

4.3.7.2.1 Preparation of drug containing media

Parallel sets of media containing two-fold dilutions of the primary anti-tubercular drugs were prepared.

Table 9. Concentrations of drugs used for resistance ratio method

S No	Concentrations of drugs used	0.5 µg/ml	1.0 µg/ml	4.0 µg/ml	8.0 µg/ml	16.0 µg/ml	32.0 µg/ml	64.0 µg/ml
1	INH	√	√					
2	RFP						√	√
3	SM					√	√	
4	EMB			√	√			

The preparations of drug solutions and drug containing media for antimicrobial susceptibility testing by resistance ratio method are given in Appendix VII.

4.3.7.2.2 Preparation of bacillary suspension

1. 0.1 ml of sterile distilled water was taken in a screw capped tube containing seven glass beads of 3 mm diameter.
2. One loopful of representative colonies from sub-cultured LJ medium was transferred to the tube by using standard 3 mm diameter loop.
3. The tube was vortexed for few minutes to produce a uniform suspension.
4. The density of the suspension was adjusted by comparing with a Mc Farland No. 1.
5. Similar procedure (from no.1 to 4) were followed for making the suspension of known (standard) strain of the tubercle bacilli i.e. $H_{37}Rv$.

4.3.7.2.3 Inoculation and incubation

1. One drop of bacillary suspension from a Pasteur pipette was spread on the surface of each eight drug containing slope of media of different concentrations as shown in table nine. Same process was applied for $H_{37}Rv$ strain.
2. All these tubes were incubated at 37°C for 4 weeks and observed at weekly intervals.

4.3.7.2.4 Interpretation of results

For all tests, growth was defined as the presence of 20 or more colonies in the drug containing media. The resistance ratio was the minimal concentration inhibiting growth of the test strain divided by the minimal concentration inhibiting growth of the standard strain, $H_{37}Rv$, in the same sets of test.

Sensitive (S)

When no growth was observed on the media containing a given drug in a given concentration.

Resistant (R)

When the growth appeared on the media containing a given drug in a given concentration in which control strain is susceptible.

4.3.8 Quality control

Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. To maintain quality control in our study, strict aseptic techniques were followed during processing of the samples. Quality control was applied during this study in various fields (Fujiki, 2001; WHO, 1998; IUATLD, 1998).

- Any leaking and broken specimen containers were discarded by autoclaving and requested for a repeated specimen.
- Smears were prepared only inside the Biological safety cabinet.
- Fresh reagents were used for the staining procedure and other biochemical tests and cytochemical staining.
- Slides were stained in batches with maximum of 12 slides per batch.
- Temperature in all instruments (Inspissator, Water bath, Refrigerator and Incubators) were checked daily and recorded in the chart.
- The inspissator was cleaned after each batch of culture media prepared. Its temperature was also checked during each period of media preparation.
- Those media with faulty coagulation, discoloration or bubbles were discarded.
- While using readymade dehydrated media, the manufacturer's instructions for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective, inhibitory, and biochemical properties of the media.
- Each batch of prepared media was checked for sterility by incubating at 37^0C for 24 hours.
- Cross-contamination of culture was avoided by using individual pipettes or loops and strict aseptic techniques.

- When each newly batch of drug media was prepared, the quality of media was checked by inoculating standard strain *M. tuberculosis* $H_{37}Rv$ suspension onto a series of drug containing media. The detailed procedure for quality check of drug containing media and interpretation table is given in Appendix VIII.

CHAPTER V

5. RESULTS

This study was conducted among the patients suspected of pulmonary tuberculosis (PTB), attending National Tuberculosis Centre (NTC), Thimi during September 2006 to June 2007. A total of 862 patients visiting NTC were included in the study.

5.1 Age and gender of the suspected PTB patients included in the study

Among the studied 862 cases, 68.2% (n=588) were male and 31.8% (n=274) were female. The age of the patients ranged from 8-95 years; with the highest percentage belonging to 21-25 years (15.5%) followed by 26-30 years (13.5%) and 31-35 years (10.6%) (Table 10).

5.2 AFB smear microscopy

Out of 862 samples only 26.2% (226) were positive for AFB in smears i.e. smear positive cases.

Among 226 smear positive samples, 76.5% (173) were male and 23.5% (53) were female patients. Thus, the highest numbers of AFB were seen in male patients (Table 11).

Table 10. Age and genderwise distribution of the patients visiting hospital

S.No.	Age group (years)	Gender of the suspected patients					
		Male		Female		Total	
		Count	% of total	Count	% of total	Count	% of total
1	5-10 yrs	2	0.2%	1	0.1%	3	**0.3%**
2	11-15 yrs	12	1.4%	14	1.6%	26	**3.0%**
3	16-20 yrs	54	6.3%	29	3.4%	83	**9.6%**
4	21-25 yrs	77	8.9%	57	6.6%	134	**15.5%**
5	26-30 yrs	80	9.3%	36	4.2%	116	**13.5%**
6	31-35 yrs	74	8.6%	17	2.0%	91	**10.6%**
7	36-40 yrs	43	5.0%	28	3.2%	71	**8.2%**
8	41-45 yrs	57	6.6%	21	2.4%	78	**9.0%**
9	46-50 yrs	55	6.4%	26	3.0%	81	**9.4%**
10	51-55 yrs	30	3.5%	14	1.6%	44	**5.1%**
11	56-60 yrs	34	3.9%	14	1.6%	48	**5.6%**
12	Above 60 yrs	70	8.1%	17	2.0%	87	**10.1%**
Total		588	68.2%	274	31.8%	862	**100.0%**

Table 11. Genderwise distribution of fluorochrome stain of the samples

S.No.	Fluorescence staining	Gender of the suspected patients					
		Male		Female		Total	
		Count	% of total	Count	% of total	Count	% of total
1	Negative	415	**48.1%**	221	**25.6%**	636	**73.8%**
2	1+	84	**9.7%**	25	**2.9%**	109	**12.6%**
3	2+	53	**6.1%**	14	**1.6%**	67	**7.8%**
4	3+	36	**4.2%**	14	**1.6%**	50	**5.8%**
Total		588	**68.2%**	274	**31.8%**	862	**100.0%**

5.3 Agewise distribution of fluorochrome stain of the samples

The maximum number of samples were collected from the age group 21-25 years and hence number of AFB positive smear was also maximum in this age group (3.8%) followed by the age group 16-20 years (3.5%), 31-35 years (3.4%) and 26-30 years (3.0%). The agewise distribution of fluorochrome stain of the sample is shown in Figure 4.

5.4 Pattern of culture results

All 226 smear positive samples and 97 smear negative samples (total-323 samples) were studied using culture technique.

Out of 323 samples, 68.4% (221) showed significant growth i.e. they were culture positive, 28.5% (92) samples were culture negative and 3.1% (10) were contaminated. Among 97 smear negative samples, 26.8% (26) samples were still culture positive and 73.2% (71) were negative. The pattern of culture result is shown in Figure 5.

5.5 Genderwise distribution of culture results

Out of 221 culture positive samples, 78.3% (173) of the samples were from male and 21.7% (48) of the samples were from female patients. Genderwise distribution of culture results is given in Table 12.

Table 12. Genderwise distribution of culture results on Ogawa medium

S.No.	Culture on Ogawa medium	Gender of the suspected patients					
		Male		Female		Total	
		Count	% of total	Count	% of total	Count	% of total
1	Smear negative not processed for culture	343	**39.8%**	196	**22.7%**	539	**62.5%**
2	1+	39	**4.5%**	15	**1.7%**	54	**6.3%**
3	2+	43	**5.0%**	13	**1.5%**	56	**6.5%**
4	3+	85	**9.9%**	16	**1.9%**	101	**11.7%**
5	4+	6	**0.7%**	4	**.5%**	10	**1.2%**
6	Contamination	8	**0.9%**	2	**.2%**	10	**1.2%**
7	Negative	64	**7.4%**	28	**3.2%**	92	**10.7%**
Total		588	**68.2%**	274	**31.8%**	862	**100.0%**

5.6 Agewise distribution of culture results

The maximum number of samples was collected from the age group 21-25 years and hence the number of mycobacterial isolates was also maximum in this age group. In this age group, the number of mycobacterial isolates was 13.6% (30); followed by the age group 16-20 years and 31-35 years, both of them containing 13.1% (29) isolates and the age group 26-30 years containing 26 (11.8%) isolates. The agewise distribution of samples is shown in Figure 6.

Pattern of culture results with respect to fluorescence staining is given in Table 13.

Table 13. Pattern of culture results with respect to fluorescence staining

S.No	Culture on Ogawa medium	Fluorescence staining									
		Negative		1+		2+		3+		Total	
		Count	% of total	Count	% of total	Count	% of total	Count	% of total	Count	% of total
1	Smear negative cases (not cultured)	539	**62.5%**	0	**0.0%**	0	**0.0%**	0	**0.0%**	539	**62.5%**
2	1+	11	**1.3%**	30	**3.5%**	6	**0.7%**	7	**0.8%**	54	**6.3%**
3	2+	6	**0.7%**	27	**3.1%**	10	**1.2%**	13	**1.5%**	56	**6.5%**
4	3+	9	**1.0%**	27	**3.1%**	44	**5.1%**	21	**2.4%**	101	**11.7%**
5	4+	0	**0.0%**	4	**0.5%**	0	**0.0%**	6	**0.7%**	10	**1.2%**
6	Contamination	0	**0.0%**	5	**0.6%**	3	**0.3%**	2	**0.2%**	10	**1.2%**
7	Negative	71	**8.2%**	16	**1.9%**	4	**0.5%**	1	**0.1%**	92	**10.7%**
Total		636	**73.8%**	109	**12.6%**	67	**7.8%**	50	**5.8%**	862	**100%**

5.7 Results of biochemical tests

All 221 culture positive isolates were niacin positive. They showed drop catalase test positive but 68°C labile catalase test negative indicating loss of catalase activity at that temperature. This confirmed the isolates to be *M. tuberculosis*.

5.8 Antimicrobial susceptibility studies

All 221 biochemically confirmed *M. tuberculosis* were subjected for susceptibility studies. The susceptibilities of these strains to each four primary anti-tubercular drugs were tested by both PR and RR methods.

Among 221 *M. tuberculosis* isolates, 49.8% (110) were susceptible to INH, 68.8% (152) were susceptible to RFP, 55.2% (122) were susceptible to SM and 74.2% (164) were susceptible to EMB by PR method. While 52.9% (117) were susceptible to INH 67.9%

(150) were susceptible to RFP, 60.2% (133) were susceptible to SM and 78.3% (173) were susceptible to EMB by RR method.

Similarly, isolates resistant to drugs were 50.2% (111) to INH, 31.2% (69) to RFP, 44.8% (99) to SM and 25.8% (57) to EMB by PR method; while 47.1% (104) to INH, 32.1% (71) to RFP, 39.8% (88) to SM and 21.7% (48) to EMB by RR method. Antimicrobial susceptibility pattern of *M. tuberculosis* is given in Table 14.

Table 14. Pattern of susceptibilities to four anti-tuberculosis drugs determined by Proportion and Resistance ratio methods

S.No.	Drugs	Proportion method		Resistance ratio method	
		Susceptible	Resistant	Susceptible	Resistant
1	INH	110	111	117	104
2	RFP	152	69	150	71
3	SM	122	99	133	88
4	EMB	164	57	173	48

The results of both methods were compared for the rates of susceptible and resistance of strains to all four drugs (Table 15). For the PR method; 94 of 221 strains (42.53%) were susceptible to all four drugs, and 127 of 221 (57.47%) were resistant to at least one drug. For the RR method; 96 of 221 strains (43.44%) were susceptible to all four drugs, and 125 of 221 (56.56%) were resistant to at least one drug. The results of susceptible and resistant rates of *M. tuberculosis* to these drugs determined by both methods were in very good agreement (k=0.852) (Thompson *et al.*, 1988).

Table 15. Comparison between the Proportion and the Resistance ratio methods for susceptible and resistance of isolates of *M. tuberculosis* to all four primary anti-tubercular drugs

RR method	PR method		Total
	Susceptible	Resistant	
Susceptible	87	9	96
Resistant	7	118	125
Total	94	127	221

K=0.852

The PR method identified 57.47% of the isolates as resistant to at least one of the four drugs. Resistance to INH and SM was the highest as both of them at 5.43%, while resistance to RFP was 0.45% and there was no EMB only resistant strain. Resistance to one, two, three and four drugs was observed in 11.31, 14.93, 14.03, and 17.19 percent of the isolates respectively. MDR was found in 29.86% of the isolates. Resistance to SM and others, INH and others, RFP and others, and EMB and others was found in 44.80, 50.23, 31.22, and 25.79 percent respectively.

The RR method identified 56.56% of the isolates as resistant strains. Resistance to INH was the highest at 5.88%, while resistance to RFP and SM were 1.81 and 4.52% respectively. There were no EMB only resistant strains. Resistance to one, two, three and four drugs was observed in 12.22, 17.65, 13.57, and 13.12 percent respectively.
MDR was found in 28.96% of the isolates. Resistance to SM and others, INH and others, RFP and others, and EMB and others was found in 39.82, 47.06, 32.13, and 21.72 percent respectively.

The resistance pattern of the isolates is given in Table 16 and Figure 8.

Table 16. Patterns of resistance of *M. tuberculosis* to primary anti-tuberculosis drugs determined by the Proportion and the Resistance ratio methods

Pattern	No. of strains			
	RR method	**%**	**PR method**	**%**
Resistance	**125**	**56.56**	**127**	**57.47**
Monoresistance to	27	12.22	25	11.31
INH	13	5.88	12	5.43
RFP	4	1.81	1	0.45
SM	10	4.52	12	5.43
EMB	0	--	0	--
Resistance to 2 drugs	39	17.65	33	14.93
SM+INH	21	9.50	25	11.31
SM+RFP	1	0.45	0	--
SM+EMB	4	1.81	1	0.45
INH+RFP	10	4.52	3	1.36
INH+ EMB	2	0.90	3	1.36
RFP+EMB	1	0.45	1	0.45
Resistance to 3 drugs	30	13.57	31	14.03
SM+INH+RFP	18	8.14	17	7.69
SM+INH+EMB	4	1.81	5	2.26
SM+RFP+EMB	1	0.45	1	0.45
INH+RFP+EMB	7	3.17	8	3.62
Resistance to 4 drugs SM+INH+RFP+EMB	29	13.12	38	17.19
MDR-TB	64	28.96	66	29.86
INH+RFP	10	4.52	3	1.36
SM+INH+RFP	18	8.14	17	7.69
INH+RFP+EMB	7	3.17	8	3.62
SM+INH+RFP+EMB	29	13.12	38	17.19
Resistance to SM and others	88	39.82	99	44.80
Resistance to INH and others	104	47.06	111	50.23
Resistance to RFP and others	71	32.13	69	31.22
Resistance to EMB and others	48	21.72	57	25.79

5.9 Agreement between Proportion and Resistance ratio methods

The percentages of agreement between the PR and the RR methods for antimicrobial susceptibility of 221 *M. tuberculosis* to INH, RFP, SM and EMB were 93.21, 93.67, 93.21 and 94.12 percent, respectively.

Table 17. Percentage agreement between the Proportion and the Resistance ratio methods for susceptibility testing of *M. tuberculosis* to each drug tested

S.No	Drugs	No. of isolates with the following results		Percent agreement
		PR method-Susceptible RR method-Susceptible	PR method-Resistant RR method-Resistant	
1	INH	106	100	93.21
2	RFP	144	63	93.67
3	SM	120	86	93.21
4	EMB	162	46	94.12

Correlation between both methods for determining susceptibilities of these strains to four drugs tested is shown in Table 18. There was high agreement between both methods when tested against INH, RFP, SM and EMB with kappa, k=0.864, 0.854, 0.861 and 0.838 respectively. Statistical comparison using Mc Nemar $\chi 2$ test showed the value 0.118, 0.791, 0.007 and 0.022 for INH, RFP, SM and EMB respectively. These values revealed that there was no statistically significant difference between both methods for determining susceptibilities to all the four drugs tested ($p>0.05$).

Table 18. Comparison between the Proportion and the Resistance ratio methods for determining susceptibility of *M. tuberculosis* to four primary anti-tubercular drugs

S.No.	Drugs	RR method	PR method		Total	K
			Susceptible	Resistant		
1	INH	**Susceptible**	106	11	117	**0.864**
		Resistant	4	100	104	
		Total	110	111	221	
2	RFP	**Susceptible**	144	6	150	**0.854**
		Resistant	8	63	71	
		Total	152	69	221	
3	SM	**Susceptible**	120	13	133	**0.861**
		Resistant	2	86	88	
		Total	122	99	221	
4	EMB	**Susceptible**	162	11	173	**0.838**
		Resistant	2	46	48	
		Total	164	57	221	

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Tuberculosis is an immense problem of the world, causing great suffering and death. It remains as the most significant cause of morbidity and mortality due to a single infectious agent in the world. In Nepal, each year 40,000 people develop active TB. Despite of the implementation of the DOTS strategy by National Tuberculosis Programme (NTP) 5,000 to 7,000 people still die from TB each year (NTC, 2007).

During this study, a total of 862 sputum samples were collected from the suspected TB patients. The numbers of male patients were 588 (68.2%) and those of females were 274 (31.8%). The age of the suspected patients in the study ranged from 8 years to 95 years. The highest number of cases belonged to the age group 21-25 years.

Out of 862 samples, 226 (26.2%) samples were smear positive for AFB and 636 (73.8%) samples were negative for AFB. the highest number of AFB positive cases were seen in male patients than in female and was observed in age group 21-25 years followed by 16-20 years and 31-35 years. This shows that the maximum numbers of TB patients are male and are in the productive age group. This finding coincides with the similar studies performed by Bhattarai *et al.* (2003).

In this study, males were found in higher percentage than females. This might be due to more exposure to external environment than females for their job and other activities, and also infected women may progress more frequently to disease and die more rapidly, leaving a cohort with a low prevalence of infection.

All 226 smear positive samples and 97 smear negative samples were processed for culture. Out of these total, 221 (68.4%) showed significant growth, 92 (28.5%) samples were culture negative and 10 (3.1%) were contaminated. Among 97 smear negative

samples, 26 (26.8%) samples were culture positive and others were negative. Among 226 smear positive samples, 21 (9.3%) samples were culture negative. The growth negativity may be due to the following reasons Toman (1979):

- In patients receiving chemotherapy, the organisms may have lost their ability to grow on culture media and be practically dead.
- Excessive decontamination procedure before inoculation.
- Tubercle bacilli may not be evenly distributed in the sample.
- The disease may be due to AFB other than *M. tuberculosis.*

According to WHO (1998), a contamination rate of 2% to 3% is acceptable in laboratories that receive fresh specimens; but it may be as high as 5% to 10% if specimens (especially sputum) take several days to reach the laboratory. In this study, the contamination rate was in the acceptable range.

For identification of isolates, the buff coloured and rough colonies appearing bread crumbs or cauliflower (rough, tough and buff colonies), no growth in less than one week (slow-grower), no pigment production and their inability to grow at 25°C were used as preliminary identification criteria. The confirmatory tests were done by niacin production and catalase test to confirm the isolates as *M. tuberculosis*.

In the treatment and control of infectious disease caused by pathogen, susceptibility test is used to select effective antimicrobial drugs. Susceptibility test is also performed to determine the changing pattern of susceptibility among pathogens to antimicrobial drugs. Since drug-resistant TB has increased in incidence and interfered with TB control programs, monitoring of drug resistance patterns is very much important to prevent MBR-TB outbreaks. So, all isolates of *M. tuberculosis* should be tested for their susceptibilities to the primary anti-tubercular drugs. The results can be used as the guidance for proper treatment. The testing may be valuable for confirmation of drug resistance in patients showing unsatisfactory response to treatment, and may be useful for identifying primary and acquired drug resistance trend in a community.

Of the conventional culture-based techniques for antimicrobial susceptibility testing, the resistance ratio (RR) and the proportion (PP) methods are commonly used. The resistance ratio method are still in use in many countries especially the United Kingdom. However, WHO has recommended the use of the proportion method to be used for determining drug susceptibility of *M. tuberculosis.*

To determine the correlation of the RR and the PR methods for susceptibility testing of *M. tuberculosis* to the four primary anti-tubercular drugs, all 221 biochemically confirmed isolates were enrolled in this study. In general, the percentages of agreement determined by both methods were high with regard to all drugs tested. This finding was concordant with similar studies done by Laszlo *et al.* (1983) which gave overall agreement of both methods higher than 95% to all drugs tested. Similarly Snider *et al.* (1981) in a large scale comparative study of drug susceptibility testing of *M. tuberculosis*, stated that a level of agreement of 90 to 95% between two tests must be considered good. This criterion reveals the good agreement rate between both methods in this study.

Both methods vary greatly in drug concentrations and interpretation of the drug resistance results. Since this study was performed by using the same inoculum size of each isolate adjusted to McFarland No. 1 for testing by both methods at the same time, no variation in inoculum size occurred. The rate of at least one or more primary drug resistance by the RR method, 125 (56.56%) was slightly less than that of the PR method, 127 (57.47%). For the single drug resistance determined by both methods, distribution rate of resistance to all drugs had no difference. The rate of two and three drugs resistance were also almost similar by both methods. But the rate of four drugs resistance in this study was slightly different between these methods. Siddiqi *et al.* (1985) showed the variations of the results have always been a problem for in vitro susceptibility testing especially at lower concentrations. Two concentrations of all drugs were used by both methods, and the high concentration had the percentage of resistance less than low concentration (result not shown). The rate of MDR-TB in this study was

similar between these methods. It was 64 (28.96%) by RR method and 66 (29.86%) by PR method.

The resistance rate was higher because most of the isolates were from relapse, after defaulted, treatment failure and chronic cases (case type not shown in the result section). Resistance was fundamentally a phenomenon linked to a large bacterial population. The far greater population of drug resistance was found in cavitary TB patients more than that of non-cavitary TB patients (Rist *et al.,* 1964).

There may be several important factors of different susceptibility results: variation in drug stability, and preparation of inoculum size. Susceptibility test results not only depend on the presence or absence of growth on the control and drug-containing media, the inoculum for each culture must also be carefully controlled (Canetti *et al.,* 1969). Furthermore, antimicrobial susceptibility test should be performed preferably with an inexpensive and relatively simple technique.

The RR method compares the resistance of the unknown strain with that of the control strain on the same batch of medium. Some workers use the $H_{37}Rv$ strain of *M. tuberculosis* as the control strain while other use three wild strains, taking their modal resistance as control. In this study, $H_{37}Rv$ strain was used as control. Smooth suspensions must be used. Large clumps of bacilli give irregular results and make reading difficult. Resistance can be expressed as the ratio of the MIC of the test strain to the MIC of the control strain in the same test. The resistant strains give the ratio of 4 or more. To determine the ratio, too many sets of media containing two fold dilutions of the drug should be prepared which is very tedious and expensive. So, in this study for each drug tested, only two concentrations were used for both test and control strain. The RR method was convenient for inoculum preparation and required a shorter time. Interpretation of the result was rather simple.

For PR method, several dilutions of the inoculum were made and both drug-free and drug-containing media were inoculated. This method was technically very difficult.

There was much risks attached to standardizing the inocula than with the RR method. However, there are several new methods e.g. E-test, Alamar blue, DNA probes and molecular finger-printing, but these methods are more expensive, require specialized equipment and highly skilled personnel. Thus, they are difficult for use in general laboratories although they provide results within 1-5 days.

While comparing the RR and the PR methods, Mc Nemar $\chi 2$ test showed no significant difference between both methods and there were very good agreement rates of the both methods when compared using kappa analysis with kappa value 0.864, 0.854, 0.861, and 0.838 for INH, RFP, SM and EMB respectively. Similar results were shown by the study done by Tansuphasiri *et al.* 2001 with kappa value 0.929, 0.621, 0.893 and 0.620 for INH, RFP, SM and EMB respectively. The closer kappa is to 1.0, the higher the accuracy of the data.

Among the tested antimicrobial agents, this in vitro testing showed EMB was the most effective drug followed by RFP. EMB is effective against drug resistant strains of *M. tuberculosis*, with bacteriostatic effect. Similarly, RFP is active against both drug sensitive and resistant strains of *M. tuberculosis*. Literature reviews and the present study clearly showed that both RFP and EMB are most effective drugs. Higher rates of resistance to INH and SM might be due to the fact that because of their low cost and wide spread use in the treatment of TB.

Alarming rate of drug resistance has been reported from developing countries like ours, which are mainly acquired resistance. According to Malla (1996), the acquired MDR-TB cases are quite high ranging from 9.6% to 66.7%. In Nepal, MDR-TB in retreatment cases was 20.5% in 2004 (WHO, 2006). The acquired resistance results due to incorrect and inadequate chemotherapy. The reasons are that the patients do not take prescribed medications with sufficient regularity and duration to achieve cure. The strategy for improving the treatment system is to implement DOTS. DOTS is our only available hope for preventing drug resistant TB. We all must come together to fight the TB epidemic and stop it at the source.

6.2 Conclusion

In this study, the highest agreement has been observed between the resistance ratio and proportion methods (with agreement rate to INH, RFP, SM and EMB of 93.21, 93.67, 93.21, and 94.12% respectively) and the correlation between both methods to the four primary anti-tubercular drugs tested was not statistically significantly different by Mc Nemar $\chi 2$ test *($p>0.05$)*. Similarly, the kappa (k) value for INH, RFP, SM and EMB were 0.864, 0.854, 0.861 and 0.838 respectively which showed good agreement between both methods.

The proportion method has been recommended by WHO for determining drug susceptibility of *M. tuberculosis* however, the resistance ratio method is also equally compatible and hence can be used for drug susceptibility testing. The proper determination of drug resistance by the proper method is helpful to minimize the spread of drug-resistant TB.

CHAPTER VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

This study was carried out at National Tuberculosis Centre (NTC), Thimi, Bhaktapur, Nepal during September 2006 to June 2007 to compare the two in vitro antimicrobial susceptibility methods (the resistance ratio and the proportion methods) for drug susceptibility testing of *M. tuberculosis.*

A total of 862 samples were collected from the suspected PTB patients during the study period. Out of total, 588 (68.2%) samples were from male and 274 (31.8%) samples from female.

Of total, 226 (26.2%) samples were positive for AFB by fluorescence microscopy. The highest number of AFB smear positive was observed in the age group between 16 years and 35 years.

Culture examination of 323 samples revealed 68.4% (221) of significant growth, 28.5% (92) were culture negative and 3.1% (10) were contaminated. All 221 culture positive isolates gave niacin production test and drop catalase test positive while 68°C labile catalase test was negative confirming the isolates to be *M. tuberculosis.*

Antimicrobial susceptibility test was performed with primary sets of anti-tubercular drugs- INH, RFP, SM, and EMB by both proportion and the resistance ratio method. Of the total 221 samples tested for drug susceptibility, by PR method 127 (57.47%) were resistant to one or more drugs whereas 94 (42.53%) were susceptible to all four drugs. While by RR method, 125 (56.56%) were resistant to one or more drugs whereas 96 (43.44%) were susceptible to all four drugs.

The highest resistance was observed with INH (50.2% i.e. 111) followed by SM (44.8% i.e. 99), RFP (31.2% i.e. 69) and EMB (25.8% i.e. 57) by PR method. And similar results was obtained by RR method with INH (47.1% i.e. 104) followed by SM (39.8% i.e. 88), RFP (32.1% i.e. 71) and EMB (21.7% i.e. 48). Multi-drug resistant isolates were 66 (29.86%) by PR method and 64 (28.96%) by RR method. This showed EMB and RFP were the must effective drugs according to susceptibility test in comparison to INH and SM.

This study showed high agreement between the RR and PR methods with agreement rates to INH, RFP, SM, and EMB of 93.21%, 93.67%, 93.21% and 94.12% respectively. The correlation between both methods for determining susceptibilities of *M. tuberculosis* to the four primary anti-tubercular drugs tested was not significantly different by Mc Nemar $\chi 2$ test ($p>0.05$). Similarly, very good agreement between both methods was found by using kappa analysis.

7.2 RECOMMENDATIONS

Finding of this study suggest the following recommendations:

1. Resistance ratio method can also be equally useful for antimicrobial susceptibility testing, though WHO recommended the use of proportion method for determining drug susceptibility of *M. tuberculosis.*
2. Performing culture of the smear negative samples could be helpful in complete and accurate diagnosis of patient.

CHAPTER-VIII

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APPENDIX I

Questionnaire for the PTB patient

S.No..... Lab No..... Date: ……....

Name: ……..…….. Age…….. Sex……...

Address: ….……...

Case type: New Relapse Chronic

Follow-up After-defaulted

Macroscopic examination: Saliva Purulent Mucopurulent

Mucoid or mucosalivary Blood stained

Microscopic examination: Positive Exact count 1+

2+ 3+

Negative

Culture: Positive Exact count 1+

2+ 3+ 4+

Negative

Contamination

Colony characteristics: ……..

Biochemical tests:

1. Catalase test:

a) Drop catalase test: Positive Negative

b) 68°C labile method Positive Negative

2. Niacin production test: Positive Negative

Antimicrobial susceptibility testing:

Proportion method				**Resistance ratio method**			
INH	RFP	SM	EMB	INH	RFP	SM	EMB

APPENDIX II

Materials used during the study

1. Equipments

Autoclave	Centrifuge
Balances-top loading and analytical	Refrigerator
Incubator	Vortex mixer
Microscope for fluorescence microscopy	pH meter
Microscope with oil immersion lens	Distillation plant
Water bath	Inspissator
Hot air oven	Homogenizer, for eggs
Pipette washer	Bunsen burner
Heater	

2. Glass wares

Graduated cylinders	Conical flasks
Pasteur pipettes	Graduated pipettes
Glass tubes, screw cap	Slides
Beakers	Petri dish
Bottles, for reagents	Filter funnels

3. Plastics

Bags	Reagent bottles
Centrifuge tubes, screw cap	Inoculating loops, standard
Plastic container, for specimen collection	

4. Safety

Biological safety cabinet, BSC II A	Centrifuge safety cups
Discard pans	Disinfectant
Protective clothing’s	Safety pippetting device

5. Chemicals and media base

Propylene glycol (propane-1, 2 diol)
Barium chloride
Hydrogen peroxide, 30% superoxol
Glycerol, reagent grade
Potassium dihydrogen phosphate
Malachite green
Sodium hydroxide, pellets
Sodium chloride, pellets
Disodium phosphate, anhydrous
Monopotassium phosphate, anhydrous
P-Nitrobenzoic acid
Alcohol, 95%
Conc. Sulphuric acid
Fuchsin, basic
Magnesium citrate
Sodium glutamate
Tween 80
Neutral red
Immersion oil
Eggs
Methylene blue
Auramine

6. Miscellaneous supplies

Beads, 3mm diameter
Pipette feeders
Racks for tubes
Staining racks
Forceps
Blotting papers
Marker
Cotton wool
Diamond pens
Slides
Thermometers
Slide driers
Aluminum foil

APPENDIX III

Composition and preparation of staining reagents

A. Fluorochrome staining reagents

i. Auramine solution

Auramine	1.0 g
Ethanol	100.0 ml

Auramine was dissolved in ethanol. ----------solution I

ii. Phenol solution

Phenol melted	30.0 ml
Distilled water	870.0 ml

Phenol was dissolved in distilled water ----------solution II

Solutions I and II were mixed and stored in tightly stopper amber bottle away from heat and light.

iii. Decolorizing solution (20% Sulphuric acid)

Concentrated H_2SO_4	200.0 ml
Distilled water	800.0 ml

Always Sulphuric acid is added to water but not water to acid.

iv. Counter stain solution (Methylene blue)

Methylene blue	1.0 g
Distilled water	1000.0 ml

About 1.0 g of Methylene blue was added in 1000.0 ml distilled water and mixed well.

B. Ziehl-Neelsen staining reagent

i. Fuchsin

Basic fuchsin	3.0 g
Ethanol	100.0 ml

Basic fuchsin was dissolved in ethanol. ---------- Solution I

ii. Phenol solution

Phenol melted	5.0 ml
Distilled water	95.0 ml

Phenol was dissolved in distilled water. ---------- Solution II

Working solution

10.0 ml of Solution I was combined with 90.0 ml of Solution II and stored in an amber bottle.

iii. Decolorizing solution (25% Sulphuric acid)

Concentrated Sulphuric acid	250.0 ml
Distilled water	750.0 ml

Always Sulphuric acid is added to water but not water to acid.

iv. Counter stain solution (Methylene blue)

Methylene blue	1.0 g
Distilled water	1000.0 ml

About 1.0 g of Methylene blue was added in 1000.0 ml distilled water and mixed well.

APPENDIX IV

Composition and preparation of digestion and decontamination reagents

i. 4% Sodium hydroxide solution

Sodium hydroxide, pellets	4.0 g
Distilled water	100.0 ml

NaOH was dissolved in distilled water with frequent shaking and sterilized by autoclaving at 121°C for 15 mins.

ii. Sterile saline

Sodium chloride, pellets	0.85 g
Distilled water	100.0 ml

NaCl was dissolved in distilled water with frequent shaking and sterilized by autoclaving at 121°C for 15 mins.

Sputum smear preparation (WHO, 1998)

1. A clean, grease free slide was taken and labeled at one end with the relevant patient number.
2. The appropriate material was transferred to the slide by using an applicator stick. Blood-specked, opaque or grayish or yellowish cheesy mucus was used for smear preparation whenever it was present.
3. The material was spread evenly over an area of size approximately 2×1 cm^2. Only one smear was prepared per slide.
4. The smear was dried at room temperature for about 15 minutes.
5. Then, it was heat fixed by passing through the flame 3-4 times.
6. These overall processes were carried inside the safety cabinet. Then, prepared smears were examined by fluorochrome staining method.

Sodium hydroxide (Modified Petroff) method for digestion and decontamination of sputum sample (Fujiki, 2001)

1. Two volumes of 4% NaOH were added to one volume of sputum specimen. The cap was tighten and shaken to digest the specimen.
2. It was left for 15 minutes without disturbing at room temperature.
3. Then, the tube was centrifuged at 3,000 xg for 15 minutes.
4. Then it was left for 10 minutes and the supernatant was poured off.
5. 15 ml of sterile distilled water was added and the sediment was resuspended.
6. The mixture was again centrifuged at 3,000 xg for 15 minutes.
7. After standing for 10 minutes to decant the supernatant, it was immediately used for the culture.

Ziehl-Neelsen (ZN) staining procedure (Fujiki, 2001)

The heat fixed slides were placed on a staining rack in batches (maximum 12), with smears facing up.

1. The slides were flooded with carbol fuchsin covering the whole surface.
2. The slides were heated with spirit cotton till steam comes off from the stain. The slides were not allowed to dry.
3. The slides were left for about 5 minutes.
4. The process was repeated 2-3 times and allowed to stand for few minutes.
5. The slides were tilted to drain off excess stain. The staining solution was washed off with a gentle stream of running water.
6. The slides were tilted to drain off excess water. Then, the slides were decolorized with 20% Sulphuric acid, until solution runs clear.
7. The slides were then washed with a gentle stream of running water and again tilted to drain off excess rinsed water.
8. 0.1% Methylene blue was poured to cover the whole surface of the slides and left for 2-3 seconds.

9. Methylene blue was then poured off and the slides were again washed with a gentle stream of running water.
10. The slides were tilted and placed on the slide rack to dry.
11. One drop of immersion oil was put on the stained smear and examined under 100X objective with 10X eye piece lens.

AFB appears red or pink coloured and the background is stained in blue colour.

APPENDIX V

Composition and preparation of culture media

A. 2% Modified Ogawa medium

i. Salt solution of 2% Modified Ogawa medium (Ingredients)

Potassium dihydrogen phosphate	2.0 g
Magnesium citrate	0.1 g
Sodium glutamate	0.5 g
Distilled water	100.0 ml

Each ingredient were weighed individually and dissolved in distilled water with frequent shaking. Then autoclaved at 121°C for 15 mins.

ii. Glycerol, reagent grade

Glycerol was added when the salt solution was warm.

iii. 2% malachite green solution

Malachite green dye	2.0 g
Distilled water	100.0 ml

4.0 ml of Malachite green solution was added to the above volume.

Preparation of whole egg homogenate

Fresh hen's eggs were cleaned by brush and soap, and then run with tap water. Then dried and wiped the outer surface with spirit cotton. After drying, the eggs were cracked with a sterile forceps into a Petri dish to check the freshness of the egg. The chalazae attached to the egg yolk were removed and the eggs were transferred to sterile blender.

The whole egg homogenate was then filtered through two layers of sterile gauze into a sterile cylinder.

Preparation of complete medium

To the above salt solution, 200.0 ml filtered egg homogenate was added and mixed well. 5.0 ml of raw medium were distributed in each tube. The tubes were laid down on the slanting bed and placed in an inspissator which has been set at 90°C before hand. The tubes were left in the inspissator at 90°C for an hour. The tubes were cooled, sterility checked and stored in the refrigerator.

B. Lowenstein-Jensen (L-J) medium

i. Salt solution of L-J medium

Ingredients	gm/litre
L-Asparagine	3.60
Monopotassium phosphate	2.40
Magnesium sulphate	0.24
Potato starch, soluble	30.00
Malachite green	0.40

37.50 g of L-J medium base (ready made powder form) was weighed and mixed by heating with 600.00 ml distilled water by taking in a 500 ml sterile conical flask. Then, it was sterilized by autoclaving at 121°C for 30 mins.

ii. Glycerol, reagent grade 12.0 ml

Glycerol was added when the salt solution was warm.

Preparation of whole egg homogenate

Fresh hen's eggs were cleaned by brush and soap, and then run with tap water. Then dried and wiped the outer surface with spirit cotton. After drying, the eggs were cracked with a sterile forceps into a Petri dish to check the freshness of the egg. The chalazae attached to the egg yolk were removed and the eggs were transferred to sterile blender. The whole egg homogenate was then filtered through two layers of sterile gauze into a sterile cylinder.

Preparation of complete medium

To the above salt solution, 1000.0 ml filtered egg homogenate was added and mixed well. 5.0 ml of raw medium were distributed in each tube. The tubes were laid down on the slanting bed and placed in an inspissator which has been set at 90°C before hand. The tubes were left in the inspissator at 90°C for an hour. The tubes were cooled, sterility checked and stored in the refrigerator.

C. Mc Farland No. 1 preparation

0.05 ml of 1% Barium chloride solution was added to 9.95 ml of Sulphuric acid with constant stirring. The Mc Farland standard was thoroughly mixed to ensure that it was evenly suspended. The tube was sealed tightly to prevent loss by evaporation and stored. The turbidity standard was then vigorously agitated on a vertex mixer before use. Standards may be stored for up to 6 months, after which, they should be discarded and new standards should be prepared.

APPENDIX VI

Composition and preparation of different biochemical reagents

A. Niacin test reagents:

i. Aniline solution

Fresh, clear colourless aniline solution	4 ml
Ethanol 95%	96 ml

Aniline was mixed with ethanol in an amber bottle and was stored in the dark in the refrigerator.

ii. Cyanogen bromide solution

Cyanogen bromide crystals	5 gm
Distilled water	50 ml

Cyanogen bromide crystals were added to distilled water in a glass beaker. The beaker was covered with foil and left for sometime at room temperature. The crystals took approximately 24 hours to dissolve at room temperature. It was poured into a tightly capped amber coloured bottle and stored in the refrigerator. It was warmed to room temperature to dissolve any precipitate formed upon cooling.

B. Catalase test reagents:

0.067M phosphate buffer solution, P^H 7.0

Anhydrous disodium phosphate	9.47 g
Distilled water	1000.0 ml

Disodium phosphate was dissolved in distilled water to provide a 0.067 M solution. ---------- Solution I

Monopotassium phosphate	9.07 g
Distilled water	1000.0 ml

Monopotassium phosphate was dissolved in distilled water to provide a 0.067 M solution. ---------- Solution II

Just before use, mixed 61.1 ml of Solution I with 38.9 ml of Solution II, by maintaining P^H 7.0

Tween 80 (10%)

Tween 80	10.0 ml
Distilled water	90.0 ml

Tween 80 was mixed with distilled water and autoclaved at 121°C for 10 mins. The tween settle during autoclaving so, it was resuspended by swirling and it was stored in the refrigerator.

Complete Catalase reagent (Tween-peroxide mixture)

Immediately before use, mixed equal parts of 10% Tween 80 and 30% hydrogen peroxide to give tween-peroxide mixture.

APPENDIX VII

Preparation of Drug solutions and Drug containing media

A. Proportion method:

1. Isoniazid (INH)

Solution I: 50.5 mg INH mixed with 10.0 ml distilled water (5000 µg/ml)
Solution II: 1.0 ml of Solution I added up to 50.0 ml distilled water (100 µg/ml)
Solution III: 1.0 ml of Solution II added up to 40.0 ml distilled water (2.5 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	8.0	0.25
L-J media (ml)	135.0	135.0
Solution II (ml)	12.0	--
Solution III (ml)	--	15.0
Distilled water (ml)	3.0	--
Total volume (ml)	150.0	150.0

2. Rifampicin (RFP)

Solution I: 20.8 mg RFP mixed with 5.0 ml Propane-1, 2 diol (4000 µg/ml)
Solution II: 4.0 ml of Solution I added up to 40.0 ml distilled water (400 µg/ml)
Solution III: 20.0ml of Solution II added up to 40.0ml distilled water (200 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	40.0	20.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

3. Streptomycin (SM)

Solution I: 21.3 mg SM mixed with 4 ml distilled water (4000 µg/ml)

Solution II: 1.0 ml of Solution I added up to 50.0 ml distilled water (80 µg/ml)

Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (40 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	8.0	4.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

4. Ethambutol (EMB)

Solution I: 34.0 mg EMB mixed with 25.0 ml distilled water (1000 µg/ml)

Solution II: 1.0 ml of Solution I added up to 50.0 ml distilled water (20 µg/ml)

Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (10 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	2.0	1.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

B. Resistance ratio method:

1. Isoniazid (INH)

Solution I: 50.5 mg INH mixed with 10.0 ml distilled water (5000 µg/ml)
Solution II: 0.1 ml of Solution I added up to 50.0 ml distilled water (10 µg/ml)
Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (5 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	1.0	0.5
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

2. Rifampicin (RFP)

Solution I: 20.8 mg RFP mixed with 5.0 ml Propane-1, 2 diol (4000 µg/ml)
Solution II: 6.4 ml of Solution I added up to 40.0 ml distilled water (640 µg/ml)
Solution III: 20.0ml of Solution II added up to 40.0ml distilled water (320 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	64.0	32.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

3. Streptomycin (SM)

Solution I: 21.3 mg SM mixed with 4.0 ml distilled water (4000 µg/ml)
Solution II: 4.0 ml of Solution I added up to 50.0 ml distilled water (320 µg/ml)
Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (160 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	32.0	16.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

4. Ethambutol (EMB)

Solution I: 34.0 mg EMB mixed with 25.0 ml distilled water (1000 µg/ml)
Solution II: 4.0 ml of Solution I added up to 50.0 ml distilled water (80 µg/ml)
Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (40 µg/ml)

Concentration in drug media (µg/ml)

Final concentration (µg/ml)	8.0	4.0
L-J media (ml)	135.0	135.0
Solution II (ml)	15.0	--
Solution III (ml)	--	15.0
Total volume (ml)	150.0	150.0

The drug containing medium 5.0 ml was dispensed into each test tube. The tubes were laid down on the slanting bed and placed in an inspissator at 90°C for an hour. The tubes were cooled, sterility checked and stored in the refrigerator.

APPENDIX VIII

Quality check of media for drug susceptibility test (IUATLD, 1998; Fujiki, 2001)

Quality control of drug susceptibility tests is best performed by titrating the standard strain $H_{37}Rv$ of *Mycobacterium tuberculosis* for each newly produced batch of drug susceptibility testing media. Minimum, median and maximum numbers of resistant bacilli by 10^6 CFU are given in the table below. If these values are exceeded then the batch is considered to be inadequate. This circumstance invalidates all results obtained with that particular batch.

0.1 ml of 1mg/ml $H_{37}Rv$ suspension, approximately 10^6 bacilli, is inoculated onto each drug containing medium. Then, incubated at 37°C for 6 weeks. The numbers of colonies for natural resistant mutants are observed.

Minimum, median, and maximum numbers of bacilli resistant to anti-tuberculosis drugs for $H_{37}Rv$ of *Mycobacterium tuberculosis* per 10^6 bacilli

Drug	Concentration (mcg/ml)	Minimum	Median	Maximum
INH	0.2	0	4	32
RFP	40.0	0	0.02	--
SM	4.0	0	7	300
EMB	2.0	100	--	1,000