CHAPTER 1

1. INTRODUCTION

Tuberculosis is a major public health problem despite the fact that the causative organism was discovered more than 100 years ago and highly effective use of chemotherapy and TB control programs available in many countries. Tuberculosis is a treatable and preventable disease (Banavaliker *et al.*, 1998).

Tuberculosis is an infectious bacterial disease caused by the bacilli called *Mycobacterium tuberculosis* (and occasionally by *Mycobacterium bovis* and *Mycobacterium africanum*), these organisms are also known as tubercle bacilli (because they cause lesions called tubercles) or acid fast bacilli.

Tuberculosis is a chronic granulomatous disease affecting human and many other animals. The disease primarily affects lungs and causes pulmonary tuberculosis (PTB). It can also affect intestine, meninges, bones and joints, lymph glands, skin and other tissues of the body (Park, 2000). Pulmonary tuberculosis is the most important form of tuberculosis, which is also called as open cases of tuberculosis. The term open tuberculosis is applied to those cases in which bacilli are detectable in the sputum *M. tuberculosis*, out of the lungs, is called extra pulmonary tuberculosis. The commonest symptoms of PTB are cough for three weeks or more, fever, other symptoms are: lethargy, lassitude, loss of appetite and weight loss. In extra pulmonary tuberculosis, symptoms depend on the organs involved (WHO, 2000).

Mycobacterium tuberculosis is usually transmitted when persons with pulmonary tuberculosis aerosolize bacteria by coughing, sneezing, speaking or singing. Susceptible person inhale aerosolized bacteria, which then can implant deep within the lung and establish infection from lungs; the organism may be disseminated to other organs by the lymphatic or bloodstream. However, novel methods of transmission have been reported, for examples nosocomial infections resulting from the use of poorly cleaned,

contaminated bronchoscopes, infection due to the aerosolization of bacteria during vigorous wound irritation, disposal or peritoneal dialysate, and autopsy and embalming procedure.

Mycobacterium tuberculosis infection occurs in an alveolar macrophage initially. The bacteria replicate within the macrophage and inducing to cytokines that initiate the inflammatory response in the lungs. Macrophages & lymphocytes migrate to the site of infection and form granuloma. The function of the granuloma is to separate the infection to prevent spread to the remainders of the lung and to other organs as well as to concentrate the immune response directly at the site of the infection. The granuloma is maintained in a persistently infected host, probably due to chronic stimulation of the immune cells, and forms the basis for tuberculosis lesions (Flynn and Chang, 2001).

WHO declared TB as a global emergency in 1993 realizing the serious public health threat posed by it. There were an estimated 8.8 million new cases of TB in 2003, with an incidence rate of 141 per 100,000 populations. The global incidence rate of tuberculosis is growing at approximately 1.1% per year. Developing countries suffer the brunt of tuberculosis epidemic. To make the global situation worse, tuberculosis has formed a lethal partnership with HIV. Nepal has an elevated annual risk of TB infection. It is estimated that about 45% of total population is infected with TB, out of which 60% are in the productive age group. Each year 44,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. Introduction of treatment by Directly Observation Treatment Short Course (DOTS) has already reduced the number of deaths; however, 8,000 – 11,000 people continue to die every year from the disease (NTP, 2004). The SAARC region accounts more than 29% of global burden of tuberculosis with 0.6 million deaths every year and 2 million new cases annually (Bam *et a1.*, 2002).

Tuberculosis is a socio-medical problem (STC, 2001). Diagnosis is performed clinically, radiologically and bacteriologically. Demonstrating bacillus in the lesions by

microscopy either by Ziehl-Neelson Staining or by Fluoro-chrome staining allows highly accurate diagnosis to be made by paramedical personnel with very little trainings, and using widely available, simple and multi-purpose equipment (Deun, 2001). Examination by bacteriological culture provides the definitive diagnosis. Different biochemical tests such as niacin test, nitrate test, urease test, catalase test etc confirm the identification in culture. Culture is sensitive technique, which detects as few as ten viable bacilli. Culture increases the number of tuberculosis cases found, often by 30-50% and detects cases earlier, often before they become infectious (WHO,1998).Culture also provides the necessary material for drug susceptibility testing. Susceptibility testing is much more technically demanding after the cultures are positive. Most of the recent advances in the laboratory diagnosis of TB have been developed nowadays for rapid culture, identification, and susceptibility systems such as Bactec Rapid Radiometric culture system, Septichek AFB system, Mycobacterium Growth Indicator Tube (MGIT), Polymerase Chain Reaction (PCR), Nucleic Acid Amplification (NAA), Fast plaque TB test etc. Similarly, Tuberculin Skin Test (TST) is used to detect infection in high-risk population. TST was originally a test for active disease but it is unsuitable for that purpose because of its limited specificity. In developed countries, radiological examination of chest is usually the first diagnostic study undertaken after history and physical examination. Diagnosis by means of radiographic examination is also unreliable because other chest diseases resemble TB on X-ray (WHO, 2002).

Drug resistant tuberculosis is a case of tuberculosis (usually pulmonary) excreting bacilli resistant to one or more anti-tuberculosis drugs. Exposure to single drug due to irregular drug supply, inappropriate preparation or poor adherence to treatment suppresses the growth of the susceptible bacilli to that drug but permits the multiplication of drug resistant organism, phenomenon called an acquired resistance. Subsequent transmission of such bacilli to other person may lead to disease, which is drug resistant from the outset, a phenomenon known as primary resistance. MDR-TB is

resistant to at least Isoniazide and Rifampicin, the main anti-tuberculosis drugs (Rijal *et al.*, 2002).

The world has developed a new framework for effective TB control and a global strategy called DOTS was introduced. DOTS strategy as one of the most cost effective health interaction and recommends that effective TB treatment be a part of the essential clinical service package available in primary health care. Governments are responsible for ensuring the provision of effective TB control through the DOTS strategy. In Nepal, DOTS strategy has been implemented since 1996 and has already reduced the number of deaths. DOTS strategy has already proven its efficacy in Nepal and will have a profound impact on mortality and morbidity (NTC 2001 / 2002).

CHAPTER 2

2. OBJECTIVES

2.1 General objective

To study the anti-TB drugs susceptibility pattern of *Mycobacterium tuberculosis* of the suspected PTB patients visiting NTC.

2.2 Specific objectives

a) To isolate and identify MTB from sputum

b) To determine the drug susceptibility pattern of MTB

c) To find out the resistance pattern

d) To find out the prevalence of TB in gender and age group

e) To determine DR / MDR based on the previous history and treatment: New / Relapse / Chronic /Follow up / Default / Treatment failure

f) To evaluate the correlation of drug resistance with habits of smoking, alcoholism and previous history of TB in family

CHAPTER 3

3. LITERATURE REVIEW

3.1 Global history

Tuberculosis is a specific chronic bacterial infection disease caused by *Mycobacterium tuberculosis* bacterium, primarily affecting lungs and pharynx causing pulmonary tuberculosis. It can also affect intestine, meninges, bones and joints, lymph glands, skin and other tissues of the body.

Tuberculosis is a disease of great antiquity and has almost certainly caused more suffering and death than any other bacterial infection. It is an ancient disease. Tuberculosis was present in Egypt from early dynastic times, perhaps as early as 3700 BC.*Mycobacterium tuberculosis* has been present in the human population since antiquity; fragments of the spinal column from Egyptian mummies from 2400 BC show definite pathological signs of tubercular decay. In ancient Hindu scripts, tuberculosis is referred to as Rograj “The King of Disease” and Rajayakshman “The disease of the Kings.”

Obviously, tuberculosis was well recognized by the time of Hippocrates (460-377 BC) who gave an excellent clinical description of the disease (Hippocrates, 1939). The term Phthisis, consumption, appears first in Greek literature. Around 460 BC, Hippocrates identified Phthisis as the most widespread disease of the times, and noted that it was almost always fatal.

Exact pathological and anatomical descriptions of the disease began to appear in the seventeenth century. In this Opera Medica of 1679, Franciscus Sylvius, the Dutch physician, was the first to identify actual tubercles as a consistent and characteristics change in the lungs and other areas of consumptive patients. He also described this progression to abscesses and cavities. Hence Sylvius deduced from autopsies that

tuberculosis was characterized by the formation of nodules, which he termed “tubercles” (Lowell, 1966).

In 1720, the English physician Benjamin Marten was the first to conjecture, in his publication "A new Theory Consumption" that TB caused by “wonderfully minute living creatures”, which, once they had gained a foothold in the body, could generate the lesions and symptoms of the disease. For the early eighteenth century, Dr Marten’s writings display on great degree of epidemiological insight.

The modern knowledge of tuberculosis started from the work of Rene Theodore Laennec (1781-1826), a French clinician, who himself was a consumptive and succumbed to the disease. In 1819, he invented the stethoscope and gave accurate description of tuberculosis lesions; he described follicular (miliary) and infiltrative (exudative) forms of tuberculosis.

In the past, tuberculosis has been referred to as the “great white scourge” and by John Bunyan as “the captain of all these men of death”.

It was Jean-Antoine Villemin (1827-1892), a French military surgeon, who clearly established the transmissible nature of tuberculosis. In 1868, Villemin published the results of a series of studies in which he convincingly demonstrated that tuberculosis could be produced in rabbits by inoculating them with tuberculosis materials from man or cattle. The disease could be passed from animals to animals and differences in virulence observed between human and bovine material (Lowell, 1966).

In 1882, March 24, Robert Koch discovered a staining technique that enabled him to see *Mycobacterium tuberculosis*, thereby, announced the discovery of tubercle bacillus and succeeded in culturing it on inspissated serum. By a large series inoculations with pure cultures of the bacillus, several generations removed from the primary one. Koch transmitted disease to many animals of different species. His classical study established without doubt that the bacillus he isolated was the cause of tuberculosis (Grange, 1990).

The acid fast nature of the organism was discovered by Ehrlic in 1882 and the present method of acid fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence the name Ziehl-Neelsen Stain.

J.L Schonlein is credited to have named the disease “tuberculosis” (Rossemblatt, 1973). The word “tuberculosis” means “a small lamp” (Dubos & Dubos, 1952).Several names have been used to refer to tuberculosis in the year gone by; acute progressive tuberculosis has been referred to as “galloping consumption”. Pulmonary tuberculosis has been referred “tubes pulmonali”.

Robert Koch mentioned that there was only one mammalian *Mycobacterium tuberculosis*. The credit of distinguishing human from bovine type lies in the work of Theobald Smith (1898). In December 1890, Koch produced tuberculosis and described “Koch’s phenomena.”

X-rays, discovered in 1895 by the professor Roentgen, were put to clinical use by 1904. The findings of radiology & bacteriology helped in developing further knowledge of the disease and correlation between them.

Calmette (1836–1933) and Guerin had been studying the effect of vaccinating animals since 1913. The Bacilli Calmette Guerin (BCG) was described as an attenuated tubercle bacillus after thirteen years of sub-culturing about 231 times. It was only in the year 1924 that BCG vaccination was used. In 1933, Calmette showed that the immunity conferred by BCG lasts for more than five years and that revaccinations are harmless.

Prior to the development of effective chemotherapy in the 1950, a variety of remedies had been used to treat tuberculosis but none showed significant efficacy. Koch’s early effort to develop an effective immunotherapy i.e. by the injection of tuberculosis was an embarrassing development of the tuberculin; it did lead to the development of the tuberculin skin test. The intradermal skin test method developed by Mantoux is still the preferred method for identifying persons infected with *M. tuberculosis*.

A few years before Koch's discovery of the tubercle bacillus, bed rest had been advocated as a treatment of tuberculosis and the sanatorium movement began in Europe and the United States (Burke, 1995).

Early in the 20th century treatment of patients with tuberculosis usually involved bed rest. A dry climate and fresh air were considered important by many clinicians and many of the Sanatoria were located in rural areas at higher elevations. Nutritional therapies, fresh air, sunlight, mental tranquility and optimism were also promoted by many (Dubos and Dubos, 1952).

The most dramatic of the therapies to emerge in the late 19th and early 20th centuries were surgical therapies.Forlanini began using artificial pneumothorax in 1892.Subsequently, other surgical techniques were developed. Although the contribution of these techniques to tuberculosis control effort remains unclear, they did provide the basis for the development of Modern Thoracic Surgery Technique.

Chemotherapy in TB cod liver oil had been used in 1822 by Schenk. Koch in 1890 found gold cyanide 1/20, 00,000 lethal to tubercle bacillus in vitro and of no value in vivo. Mollgaard (1924) reintroduced gold in the form of a thiosulphate as sanocrysin; copper salts were used in 1894 in France. In 1927, Perey Moxcey introduced an antimony preparation to be used through the intramuscular route. In the modern period, the search for chemotherapeutic drugs was activated by Domagk's introduction prontosil; promin, promizole, sulphetron etc have been found useful against the tubercle bacillus. All these were found to extremely toxic and given up.

Following Koch's discovery of the tubercle bacillus, an interest in chemotherapy of the disease developed. None of the drugs studies in animals and in man showed great promise. However, until the discovery of Streptomycin by Wasksmann and coworkers in 1944.Trails by the British Medical Research Council (MRC), the United States Public Health Service (USPHS), and the US veterans Administration – Armed forces cooperative Trait Group confirmed its efficacy but drug resistance emerged as a series

drawback. Therefore, in an effort to prevent resistance in 1948, the MRC undertook a successful trail of combined streptomycin, para-amino salicylic acid (PAS) therapy.By 1952, isoniazid had become an important part of the initial treatment regimen. It was not until the early 1960s that MRC trail settled the optimal duration of treatment 2 years. A number of trails conducted during the 1950s and 1960s also demonstrated that treatment could be effectively given on an outpatient basis and that hospitalization and bed rest were unnecessarily.

Another major advance in chemotherapy in the 1960s was the discovery that therapy could be just as effective when given intermittently (two or three times weekly) as when given daily. Supervision of intermittently administered therapy is much easier and less costly than supervision of daily. The next major development, Short Course chemotherapy- usually understood to mean treatment regimens of 9 months or less – was in the 1970s. Short-course regimes with high cure rates became possible only after the introduction of rifampicin with the introduction of drugs, the chemotherapeutic armamentarium for TB included two bacterial drugs – rifampicin and isoniazid and it was subsequently shown that high cure rates could be obtained by giving both these drugs for only 9 months. The development of successful 6 month treatment regimes occurred when pyrazinamide (PZA) was added. This drug was first introduced in 1952 but not widely used because of hepatotoxicity. However, by using lower dosages and limiting the duration of treatment with PZA, the MRC Tuberculosis unit, under the leadership of professor Wallace Fox, was able to achieve results as good as, or better than, the best regimes of longer duration.

3.2. Tuberculosis disease

Tuberculosis is a disease of worldwide prevalence and is responsible for more serious illness than any other infection disease. Despite the availability of effective chemotherapy, it is still a major public health problem in the most countries of the world (Stewart and Beswick, 1977). World Health Organization (WHO) declared TB as

a global emergency in 1993 realizing the serious public health threat posed by tuberculosis. Tuberculosis causes more deaths than any other infections agent. About one third of the world's population i.e.1.7 billion people infected by *Mycobacterium tuberculosis*. Every year 2 million people die from TB, it means that one dies of TB every 15 seconds. In 1996, there were approximately eight million new cases of tuberculosis with three million deaths. TB is the leading cause of death due to a single infectious agent worldwide. 95% of tuberculosis cases and 98% of tuberculosis deaths are in the developing countries. Deaths from tuberculosis comprise 25% of all avoidable deaths in the developing world. 75% of the tuberculosis cases occur in the age group 20-49 year, which represents men and women in their most productive age. 27% of the world's estimated TB cases are from the SAARC region. In SAARC region, 5 million active TB cases, 2.5 million people TB every year and 0.6 million deaths from TB every year (NTP, 2002/2003).

The main reasons for the increasing global tuberculosis burden are of the following – poverty in various populations, not only in developing countries but also in inner city population in developed countries, changing demographics, with increasing world population and changing age, structure, insufficient and inadequate health coverage of the population, especially in poor countries and of vulnerable groups of the population in all countries, neglect and under finding of TB control program with inadequate cases detection, inadequate case management and poor cure rates in several countries mainly in Asia and the impact of HIV epidemic (WHO, 2002).

The WHO tuberculosis control policy package called the "DOTS strategy" represents an organizational framework for effective utilization of the existing tools for diagnosis and treatment. The five main components of the WHO tuberculosis control policy are: governmental commitment, case detection by passive case finding and microscopy smear examination; directly observed treatment with short course chemotherapy regimens administrated to at least all smear positive cases of TB; regular drug supply; and a monitoring system for supervision and evaluation. In countries applying the WHO

tuberculosis control policy, there has been a trend towards improvement in the detection of infectious cases (smear positive) and towards achieving cure and success rates of between 80 to 90% of detected cases.

Approximately 10% of individuals with a latent infection later progress to active disease. About half of this group do so in the first two years after the initial infection and the remaining half develop active disease at less predictable times during the remaining years of life.3% to 5% immuno-competent individuals develop TB within 1 year, once infected and an additional 3 to 5% develop TB during the remainder of their life time. The majority of individuals who have TB in their body are able to mount an effective immune response that encapsulates these organisms usually for the rest of the host's life thus preventing the progression from infection to disease (Lauzardo and Ashken, 2000).

Associated with the increased incidence of TB, there was a substantial increase in MDR-TB. The treatment of MDR-TB has been extremely difficult. The most likely reason for the poor response of MDR-TB to therapy is that INH and RMP are the two most potent mycobactericidal agents available. The specific therapy of MDR-TB depends on the exact pattern of the drug susceptibility observed for the infecting organism.

In 1997 the World Health Organization (WHO), the International Union Against Tuberculosis and Lung Disease (IUATLD) and partners world-wide released the first report of the global project on anti-tuberculosis drug resistance.The data generated in this report were reinforced in a recently published second report. Directly observed treatment short-course (DOTS), the WHO strategy for TB control cures virtually all patients with drug-susceptible TB and some drug resistant TB through the administration of short-course chemotherapy with first-line drugs.

However, patients with multidrug-resistant tuberculosis (MDR-TB) to at least isoniazid and rifampicin are more likely to fail short-course chemotherapy. In recent years there

has been encouraging evidence that patients with MDR TB can be cured with appropriate management based on second-line drugs. Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs and reliable assessment of drug resistance is an essential prerequisite for appropriate use. Treatment is prolonged and significantly more expensive. Accurate laboratory drug susceptibility testing (DST) data to second-line drugs will support clinical decision making and help to prevent the emergence of further drug resistance in patients with MDR TB. In order to meet the challenges posed by MDR TB, the WHO established the DOTS-Plus initiative to assess the feasibility and cost-effectiveness of using second-line drugs to manage patients with MDR TB primarily in middle and low-income countries. Reliable DST, including second-line drug testing, is a basic requirement of the DOTS-Plus strategy.

Based upon DOTS, DOTS-Plus is a comprehensive management strategy under development and testing that includes the five tenets of the DOTS strategy. DOTS-Plus takes into account specific issues (such as the use of second-line anti-TB drugs) that need to be addressed in areas where there is high prevalence of MDR-TB. Thus, DOTS-Plus works as a supplement to the standard DOTS strategy. By definition, it is impossible to conduct DOTS-Plus in an area without having an effective DOTS-based TB control programme in place.

DOTS-Plus is not intended as a universal strategy, and is not required in all settings. DOTS-Plus should be implemented in selected areas with moderate to high levels of MDR-TB in order to combat an emerging via the GLC review process, DOTS-Plus is being implemented in Bolivia, Costa Rica, Estonia, Haiti, Karakalpakstan (Uzbekistan), Latvia, Malawi, Mexico, Peru, Philippines and the Russian Federation (Arkhangelsk, Ivanono, Tomsk and Orel Oblasts). More recently, DOTS-Plus projects have also been approved in Georgia, Honduras, Jordan, Kenya, Kyrgyzstan, Lebanon, Nepal, Nicaragua, Romania and Syria.

3.3 The genus *Mycobacterium*

3.3.1 Definition

The only genus in the family Mycobacteriaceae. Straight or slightly curved rods, but coccobacillary, filamentous and branched forms also occur. Cells are Gram positive (through not easily stainable by this method), acid fast, non-motile and non-sporing. Some strains produce yellow pigment in the dark or after exposure to light; aerobic or microaerophilic. Acid is produced from sugars oxidatively; nutritional requirements and temperature range of growth vary considerably. Two major subdivisions are recognized – rapid growers and slow growers. Cell walls contains large amounts of lipids. The germ is distinguished by characteristics antigenic patterns and mycolic acid structures. G + C contains of DNA 66-72 mol % (except *Mycobacterium leprae* which has 55 mol %) (Grange, 1990).

The generic name of *Mycobacterium* is ("fungus bacterium") proposed by Lehmann and Neumann (1896) in reference to the mouldlike pellicle formed by *M. tuberculosis* on liquid media. The genus contains over 50 well defined species. Though essentially a genus of free living saprophytes, the mycobacterium include the causative agents of tuberculosis, leprosy and chronic hypertrophic enteritis (Johne's disease) of cattle (Grange, 1990).

An important character of the mycobacteria is their ability to resist decolorization by a weak mineral acid after being strained by arylmethane dye – acid fastness – but the genus is more accurately defined by the chemical structure of its mycolic acids (Minnikin, 1982) and its antigenic structure (Grange, 1990). The cultivable members of the genus are divisible into two major groups, the slow growers and rapid growers, which also differ in antigenic structure and in DNA relatedness.Mycobacteria are unique in synthesizing two distinct classes of iron-chelating agents – the mycobactin and exochelins (Grange, 1990).

3.3.2 *Mycobacterium* species

The genus *Mycobacterium* is very well classified as a result of extensive studies undertaken by the International Working Group on *Mycobacterium* Taxonomy (Wayne and Kubica, 1986). The Approved Lists of Bacterial Names includes four mycobacterial species and several other have been described or reintroduced subsequently. With few exceptions, each species is a well defined and distinct taxonomy entity and specification by mumuical or Adansonian taxonomy correlates closely with that obtained by antigenic analysis and DNA relatedness .Difficulties arise in the case of the *M. tuberculosis* group (*M. tuberculosis, M bovis, M. africanum & M. microti*) which are so closely related that they should really be considered as variants of one species and the *M. avium* group *(M. avium, M. intracellulare, M. paratuberculosis and M. lepraemurium)* which could likewise be regarded similarly.

Currently, there are 71 recognized or proposed species in the genus *Mycobacterium*. These species produce a spectrum of infections in humans and animals ranging from localized lesions to disseminated diseases. Many species are also found in water and soil. Four very closely related species are responsible for mammalian tuberculosis: *M. tubersulosis*, (Human tubercle bacillus), *M. bovis* (Bovine tubercle bacillus), *M. microti* (Vole tubercle bacillus) and *M. africanum* (intermediate in form between human and bovine types) (Chakraborty, 2001).

Classification of Mycobacteria:

I. Strict pathogens

a) *M. tuberculosis* complex

M. tuberculosis – human type

M. bovis – bovine type

M. africanum – human type

M. microti – muarine type

b) Lepra bacilli

M. leprae – causing leprosy in human

M. leprae murium – causing rat leprosy

c) Other animal pathogens

M. microti – murine type

M. paratuberculosis – Johne's bacillus

M. ulcerans

M. balnei

II. Atypical mycobacteria

Runyon Group I – Photochromogens

Runyon Group II – Scotochromogens

Runyon Group III – Non - chromogens

Runyon Group IV – Rapid growers

III. Saprophytic Mycobacteria (non-pathogenic)

1. M. smegmatis – Present in smegma, thermophiles grow at 52^{o}C

2. M. phlei – present in grass

3. M. stercoris – present in dung

4. M. thermoresistible

Table 1. Approved mycobacterial names arranged alphabetically

M. africanum
M. asiaticum

M. aurum
M. avium
M. bovis
M. chelonei
M. chitae
M. duvalii
M. farcinogenes
M. flavescens
M. gadium
M. gastri
M. gilvum
M. gordona
M. haemophilum
M. intracellulare
M. kansasii
M. komossene
M. leprae
M. lepraemurium
M. malmoense
M. marinum
M. microti
M. neoaurum
M. nonchromogenicum
M. parafortaitum
M. paratuberculosis (Johne's bacillus)
M. phlei
M. scrofulaceum
M. senegalense
M. simiae
M. smegmatis
M. szulgai
M. turae
M. thermoresistible
M. triviale
M.tuberculosis
M. ulcerans
M. vaccae
M. xenopi

From Skerman *et al.*, 1980

3.3.3 Morphology of *M. tuberculosis*

M. tuberculosis is straight or slightly curved rod usually 1-4µm×0.3-0.6µm in size. It may be arranged singly or small clumps. It is non-motile, non-sporing and non-capsulated, acid-fast and Gram positive bacterium. Cells of *M. tuberculosis* are often arranged is 'serpentine cords' & it demonstrate beaded or barred staining. Non-acid fast rods and granules from young culture are also reported and when they are injected into susceptible animals, they produce tuberculosis. Perhaps these granules are non-acid fast form of tubercle bacilli. These bacilli are called Much's granules (Satish Gupte, 1999).

Electron microscopy shows that mycobacterium possess a relatively thick cell wall about 20nm across. It often appears to be separated from the cell membrane by thin electron-transparent zone, but this may be an artifact. The cell membrane is seen as a bilayer and frequently shows infoldings or mesosomes, particularly at the point of cell division. The surface of the cell is often covered by a diffuse capsule of fibrillar material composed of mycosides. The cytoplasm contains well defined nuclear body, dense granules composed of polyphosphates and electron-translucent vacuoles which are probably lipid storage bodies (Grange, 1990).

3.4 Characteristics of *Mycobacterium*

3.4.1 Antigenic structural characteristics

The mycobacterial antigens may be broadly classified as:

i) cytoplasmic (soluble) or cell wall lipid-bound (insoluble)

ii) according to their chemicals structure (carbohydrate or protein) or

iii) by their distribution within the genus

Antigens have been extensively used to classify, identify and type the mycobacteria.

Soluble antigens

Up to 15 lines of precipitation are demonstrable when ultrasonicates of mycobacteria are tested against homologous antisera by immunoelectrophoresis or double diffusion in agar gel while up to 90 antigens are demonstrable by the more sensitive techniques of crossed immunoelectrophoresis.Soluble antigens are divisible into 4 major groups ;those common to all mycobacteria (group I); those occurring in slowly growing species (group II), those occurring in rapidly growing species (group III) and those unique to each individual species (group IV). This antigenic distribution suggests that the mycobacteria evolved from a common ancestral form and that there was an early division of the germs into the slow and rapid growers.

Immunoelectrophoretic analysis of culture filtrates of *M. tuberculosis* revealed 11 arcs of precipitation which were numbered for reference purposes. Antigens 1, 2 and 3 are respectively, arabinomannans, arabinogalactans and glucans that are distributed throughout the genes; antigens 6, 7 and 8 are widely distributed proteins while antigen 5 is a protein of mol. wt. 28,500-35,000 and is specific for *M. tuberculosis*. A more elaborate reference system based on 30 numbered antigens of BCG demonstrable by two-dimensional polyacrylamide gel electrophoresis was proposed by Closs *et al.*, (1980). Several of these antigens have been further characterized.Some have been shown to be enzymes.The development of ‘genome libraries” and DNA, cloning techniques now permits virtually unlimited amounts of pure peptide antigens to be obtained, even from the non-cultivable leprosy bacillus. A number of antigens derived from *M. tuberculosis* & *M .leprae* genomic libraries are recognizable by murine monoclonal antibodies.

Immunological studies on mycobacteria have largely employed antigens derived from disrupted bacterial cells, but those actively secreted by living cells may be more relevant to protective immunity or immunopathology. Secreted antigens may be identified by cultivating mycobacteria for a few days in [^{35}S] methionine, separating

antigens in the culture supernates by high resolution electrophoresis and visualizing them by autoradiography. These actively secreted antigens form only a small proportion of these released from old cultures by autolysis.

Insoluble (agglutination) antigens

Mycobacteria that form stable smooth suspensions may be identified or typed by agglutination tests. The agglutination of the *M. avium-intracellular* group has been studied extensively by Schaefer and his colleague. These serotypes are identifiable in several other species but not, unfortunately, in *M. tuberculosis* which is rough and readily autoagglutinates. The responsible antigens have been identified as the sugar moieties on mycosides; these include the major antigenic phenolic glycolipid of *M. leprae*.

3.4.2 Genetic characters

The genomes

Mycobacterial chromosomes were found to have mol. wt. of (2.5 – 5.55) $\times 10^9$, with those of the major pathogens, *M. tuberculosis* and *M. leprae* being at the lower end of the range .DNA pairing confirms the species boundaries as determined by other methods and also reveals a low homology between slowly growing and rapidly growing strains, supporting the serological evidences that these are evolutionary divergent subgenera. Similar technology has also been used to produce ‘DNA probes’ both for detecting mycobacterial genomes in sputum and for identifying strains.Mycobacterial DNA is digestible by a range of commercially available restriction endonucleases.

Mutation

Mutation occurs naturally at a low frequency and is inducible at a much higher frequency by UV light, ϒ-radiation and chemical mutagens. From the clinical point of

views, the most important mutational changes are those affecting susceptibility to antibacterial agents.

Genetic transfer

Genes have been transferred between mycobacteria only with difficulty and only in a few rapidly growing strains.

Resistance

M. tuberculosis is more resistant to drying and chemical disinfectants. Temperature 60^{o}C for 20 minutes can kill it. Moist heat at 100^{o}C kills it readily. In sunlight the culture may be killed in 2 hours. In sputum it survives 20-30 hours even in sunlight. It is killed by tincture of iodine in 5 minutes and by 80% ethanol in 2 to 10 minutes. Phenol solution (5%) kills it in 24 hours.

3.4.3 Cultural characteristics

Tubercle bacilli are aerobes and will not grow in the absence of oxygen, even 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 sometime may be delayed up to 6-8 weeks. Optimum temperature is 37^{o}C and growth does not occur below 25^{o}C or above 40^{o}C. Optimum pH is 6.4 to 7.0. They grow only in especially protein enriched media containing egg, asparagines, potatoes, serum and meat extracts (Forbes *et al.*, 2002).

3.4.3.1 Liquid medium

Liquid media are not generally employed for routine cultivation but are used for sensitivity test, biochemical tests and preparation of antigens and vaccines. The bacilli

grow either on surface as pellicle or as floccules throughout the medium due to hydrophobic nature of their cell wall (lipid). Virulent strains often grow as twisted rope like colonies called as serpentine cords. Dubo's medium containing casein hydrolysate, bovine serum albumin, asparagines and certain salts with Tween 80 is a widely used as liquid medium. Tubercle bacilli grow more quickly in liquid medium than on solid media. Liquid media generally used are Dubo's medium, Middlebrook 7H9 broth and Bactec 12B medium.

3.4.3.2 Solid medium

Solid media are mainly used for routine culture for primary isolation. Solid media are of two major types: Egg based Solid media and Agar based Solid media. Egg – Based solid media are Lowenstein-Jensen (LJ), Ogawa, Petragnam or Dorset, Wallstein and Middlebrook 7H10 and Middlebrook 7H11. The distinctive colony characteristic of *M. tuberculosis* in LJ medium or Ogawa medium is rough, tough and butt colony.

3.5 Mode of transmission

Tuberculosis is transmitted mainly by droplet infection and droplet nuclei generated by sputum positive patients with pulmonary tuberculosis by coughing, sneezing and talking. To transmit infection, the particles must be fresh enough to carry a viable organism. The airborne particles (droplet nuclei) 1-5µm in size are kept "suspended" by normal air current infection occurs when a susceptible person inhale the droplet nuclei. One air borne particle contains 1-10 bacilli. Patients who excrete 10,000 or more tubercle bacilli per ml of sputum are the main source of infection to other (Groothius and Yates, 1991). Coughing generates the largest number of droplet nuclei. Transmission generally occurs indoors, where droplet nuclei can stay in the air for a long time. The frequency and vigor of cough and the ventilation of the environment influence transmission risk of exposure – the concentration of droplets nuclei in contaminated air and the length of the time he breathes that air (WHO, 1996). Tuberculosis is not transmitted by fomites, such as dishes and other articles used by the

patients. Sterilization of these articles is, therefore, of little or no value. Patients with extrapulmonary tuberculosis or smear negative tuberculosis constitute a minimal hazard for transmission of infection (WHO, 1996).

3.6 Risk of infection

An individual risk of infection depends on the extent of exposure to droplet nuclei and susceptibility to infection. The risk of infection of a susceptible individual is therefore high with close, prolonged, indoor exposure to a person with sputum smear positive PTB. The risk of transmission of infection from a person with sputum smear negative PTB is low and with extra pulmonary TB is even lower (NTP, 1998).

Tubercle bacilli are a necessary, but not a sufficient to cause of tuberculosis. The risk of becoming infected is largely exogenous in nature, determined by the characteristics of the source case, environmental and duration of exposure, the risk of developing tuberculosis given that infection has occurred is largely endogenous, determined by the integrity of the cellular immune system (Rieder, 1995).

Incubation period

The time from receipt of infection to the development of a positive tuberculin test ranges from 3 to 6 weeks, and thereafter, the development of disease depends upon the closeness of contact, extent of the disease and sputum positivity of the source case (dose of infection) and host-parasite relationship. Thus the incubation period may be weeks, months or years (Park, 2000).

3.7 Predisposing factors in the development of tuberculosis

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 (Groothius and Yates, 1991). As immunity wanes through aging or immune suppression, the dormant

bacteria become reactive causing an outbreak of disease often many decades after the initial infection. Immunosuppression due to disease of drug therapy is a major predisposing factor for the development of mycobacterial disease (Groothius and Yates, 1991). HIV infection appears to increase the risk greatly and shorten the interval for the development of TB disease (EDCD, 2003).

3.8 Pathogenesis of TB

The tubercle bacillus owes its virulence to its ability to survive within the macrophage rather than to the production of a toxic substance. The immune response to the bacillus is of the cell-mediated type which, depending on the type of T helper cells involved, many either lead to protective immunity and resolution of the disease or to tissue destroying hypersensitivity reactions and progression of the disease process (Greenwood *et al.,* 2002). The virulence of tubercle bacilli appears to be related to their ability to survive within macrophages. Tubercle bacilli inhibit the fusion phyzosome with lysosome, by a mechanism that is poorly understood. The bacilli secrete several compounds (ammonium ions, polyglutamic acids, cyclic Amp & sulpholipids) that affect cell membranes, but their role in vivo is uncertain. It has been suggested that the alternation 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, 1994).

The nature of the immune responses following infection changes with time so that human tuberculosis divisible into primary and post primary forms with quite different pathological features.

3.9 Primary pulmonary tuberculosis

The primary phase of *M. tuberculosis* infection begins with inhalation of the mycobacteria and ends with T-cell mediated immune responses that induce hypersensitivity to the organisms and controls 95% of infection. Droplets nuclei that are

inhaled into the lungs are so small that they avoid the mucobacillary defence of bronchi and lodge in the terminal of the lungs (WHO, 2004). Most often in the periphery of one lung, inhaled *M. tuberculosis* is first phagocytosed by alveolar macrophage and transported by the cells to miliary lymph nodes. Naïve macrophages are unable to kill the mycobacteria, which multiply, lyse the host cell, infect other macrophages, and sometimes disseminated through the blood to other parts of the lungs and elsewhere in the body. In most of infected individuals, cell-mediated immunity develops 2-8 weeks after infection. Activated T-lymphocytes and macrophages form granulomas that limit further replication and spread of the organism (Schlunger and Rom, 1998). The development of cell mediated immunity against *M. tuberculosis* is associated with the development of a positive result in the activated T cells interact with macrophages in three ways:

First

$CD4^+$ helper T cell secretes interferon-ϒ, which activates macrophages to kill intracellular mycobacteria through reactive nitrogen intermediates, including NO, NO_2 and HNO_3. This is associated with the formation of epitheloid cell granualomas and clearance of the mycobacterium.

Second

$CD8^+$ suppressor T cell lyses macrophage infected with mycobacteria through Fas independent, granule development reaction and kill mycobacteria.

Third

CD 4-CD-8-T cells lyse macrophage in a Fas dependent manner, without killing mycobacteria. Lysis of macrophage results in the formation of caseating granulomas. Direct toxicity of the mycobacteria to the macrophages may contribute to the necrotic caseous centers. Mycobacteria cannot grow in this acidic, extracellular environment

lacking in oxygen, and so the mycrobacterial infections controlled. The ultimate residual of the primary infection is a calcified scar in the lung parenchyma and in the hilar lymph node, together referred to the Ghon complex.

3.10 Post-primary tuberculosis

Some individuals are reinfected mycobacteria,reactivate dormant disease or progress directly from the primary mycobacterial lesion into disseminated disease.This may be because the strain of mycobacteria is particularly virulent or the host is particularly susceptible .Granulomas of secondary tuberculosis most often occurs in the apex of the lungs but may be widely disseminated in the lungs,kidneys,menings,marrow and other organs.These granulomas which fail to contain the spread of the mycobacterial infection are the major cause of tissue damage in tuberculosis and are a reflection of delayed type hypersensitivity. Two special features of secondary tuberculosis or post-primary tuberculosis are caseous necrosis and cavities; necrosis may cause rupture into blood vessels, spreading mycobacteria throughout the body, and break into airways, releasing infectious mycobacteria in aerosols.

3.11 Extra pulmonary tuberculosis

Most, if not all, extra pulmonary lesions results by haematogenous spread of the organisms from a primary focus which is not always detected (Chakraborty, 2001).TB can affect any organ or tissue of the body. There are many types of extra pulmonary TB. These include glands, intestinal, pleurisy, miliary meningitis, bones, urogenital, skin and eye tuberculosis (NTP,1996).The highest rate of infection of extra pulmonary tuberculosis cases in immuno compromised states associated with old age,renal failure(including dialysis and transplant patients),cirrhosis, malnutrition, hematologic malignancies and HIV/AIDS. Patients usually present with constitutional features (fever, night sweat, weight loss) and local features related to the site of diseases. The local features related to the site of diseases are similar in adults and children. Many

patients with extra pulmonary tuberculosis also have co-existent pulmonary TB (WHO, 1997).

3.12 Immunology of tuberculosis (Host defence mechanism)

Man and animals are known to possess natural resistance to TB.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 mycobacteria through the air passages to the lungs. Various physiological factors also prevent the infection. Some of which are antimycobacterial substances in normal urine. A stable dialyzable in urine inhibit *M. tuberculosis*.

Human displays native immunity to tuberculosis with substantial variation (Hasleton, 1996). The different manifestations of infection with tubercle bacilli reflect the balance between the bacilli and host defence mechanisms.Tradionally, protective immunity to TB has been ascribed to T-cell mediated immunity, with CD 4+ T cells playing a crucial role. Elimination of *M.tuberculosis* infection mainly depends on the success of interaction between infected macrophages and T lymphocytes.CD4+ T cells exert their protective effect by the production of cytokines, primarily Y 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.

Recent immunological and genetic studies supported the longstanding notion that innate immunity is also revelant in tuberculosis.Phagocytic cells play a key role in the initiation and direction of acquired T-cell immunity by presentation of mycobacterial antigens and expression of stimulatory signals and cytokines. Toll-like receptors seem to play a crucial role in immune recognition of *M. tuberculosis*.Through toll-like receptors(TLRs), *M. tuberculosis* lysate or soluble mycobacterial cell wall associated lipoproteins induce production of IL-12,a strong inflammatory cytokines.Hence,TLRs play an important role in innate immunity. Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and production of cytokines, which in itself

induce further activation and cytokine production in a complex process of regulation and cross regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections. The interplay between *M. tuberculosis* and human-host determines the outcome after infection.

3.13 Diagnosis of pulmonary tuberculosis

3.13.1 Clinical diagnosis

Clinically, pulmonary tuberculosis is chiefly present with persistent cough for 3 or more weeks, haemoptysis, shortness of breath and chest pain, loss of appetite and loss of weight, malaise, fatigue, night sweat and fever (Enarson and Bam, 2001; WHO, 1997).The disease in the 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 obstruction of the bronchi and emphysema. Clinical signs and symptoms develop in only a small proportion (5-10%) of infected healthy people.

3.13.2 Radiological diagnosis

Chest X ray can also help in the detection of the pulmonary tuberculosis but they don't allow etiological diagnosis.X-ray suggesting tuberculosis include upperlobe infiltrate, cavity infiltrate and hilar or paratracheal adenopathy.In many patients with primary progressive tuberculosis and those with HIV infection radiographic findings are subtler and can include lower lobe infiltrates or a miliary pattern. Miliary lesions, which are small granulomas, resemble millet seeds spread throughout the lung fields (Bloom, 1994).

3.13.3 Laboratory diagnosis

3.13.3.1 Microscopic examination

This is a rapid and simple method to detect cases of pulmonary tuberculosis disease that are a source of infection. The reliability, cheapness and ease of direct microscopic examination has made it the number one case-finding method all over the world. It enables us to discover epidemiologically most important cases of pulmonary tuberculosis.

AFB microscopy allows for highly accurate diagnosis to be made by paramedical personnel with very little training, and using widely available, simple and multipurpose equipment (STC, 2001).The main value 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 training personnel and good microscopes and sensitivity ranges from about 25% to 75%.

The microscopy is carried out by utilizing acid fast property of mycobacteria. The acid-fast staining procedure depends on the ability of mycobacterium to retain dye even when treated with mineral acid or an acid-alcohol solution (Bloom, 1994).

Presently, two methods-Ziehl Neelsen and Fluorescent method are generally used to observe the acid fast bacilli by microscopy.

In ZN staining technique, heat fixed smears of specimen are flooded with solution of Carbol fuchsin (a mixture of basic fuchsin and phenol) and heated until steam rises. After washing with water, the slide is flooded with a dilute mineral acid (eg.HCl) and after further washing; a green or blue counter stain is applied. Red bacilli are seen against the constrating background colour. In some method, acid is diluted in 95% ethanol rather than water.

Fluorescence microscopy was introduced by Hagemann (1937), who originally used berberine sulphate as the dye but later (1938) recommended auramine.Tryant *et al.* (1962) used two arylmethane dyes, auramine O and auramine B together.This combined

staining method detect acid-fast bacilli. In fluorochrome staining, flooded smear with auramine, rinse with water and decolorize with 3% acid alcohol and again rinse with 3% acid alcohol and again rinse with water and then counterstain. The acid fast organisms appear as fluorescent rod, yellow to orange (the colour may vary with the filter system used).If daily examinations exceed 50,fluorescent staining method is to be preferred (WHO,1998).

Other types of staining procedures used in the laboratory for rapid detection and confirmation of AFB are Kinyoun (Cold staining) and Modified Cold Stain.The classic carbon fuchsin (ZN) requires heating the slide for better penetration of the stain into the mycobacterial cell wall, hence it is also known as the Hot Stain procedure. Kinyoun acidfast is similar to ZN but without heat, hence the term Cold Stain. Kinyoun requires the high concentration of basic fuchsin and phenol or the addition of a detergent, thereby avoiding the need for heat. In this study, a new, cheaper, safer and easier staining method named as Modified Cold Stain is described for the demonstration of AFB (Forbes *et al.*,1998).Though not popular, this technique is an improved acidfast staining technique, which is far easier, safer, rapid and invasive technique. The technique was modified and simplified by eliminating heating step and combining the stage of decolourisation and counter staining .When acidfast organisms are observed on a smear, result must be quantified to be meaningful because this quantitation estimates the number of bacilli being excreted; the extent of a patient's infectiousness can be assessed for clinical and epidemiological purpose (Forbes *et al.*, 1998).

The microscopy method detects 5,000-10,000 bacteria per ml with sensitivity range between 46-78% and specificity is virtually 100% depending on the source of the sample and the mycobacterium involved. Still, smear negative, culture positive results often occur. However, the increased sensitivity of microscopy from 51% to almost 100% by introducing the cytocentrifugation for the concentration of sputum samples has been reported. The sensitivity tends to be the highest in the patients who have

cavitary disease and the lowest in the patients who have week cough or less advanced disease.

3.13.3.2 Culture method

TB can be definitely diagnosed by isolating the causative organisms in pure culture. So the culture remains "gold standard" for diagnosis of tuberculosis. Diagnosis of TB culture method is most sensitive than AFB staining method and can be reliably find mycobacteria when they are present in a concentration of about 10^3 organisms/ml of specimen. According to Yeager *et al.,* 1967, cultural technique may detect as few as 10-100 organisms/ml of sputum. As a result, the sensitivity of culture is excellent ranging from 80%-93%Moreover, the specificity is quite high at 98%.Culture increases the susceptibility for diagnosis of *M.tuberculosis* and allow specification, drug susceptibility testing, and if needed, genotyping for epidemiologic purposes.

Recovery of *M. tuberculosis* from clinical sample consumes more time than for normal pathogenic bacteria; requiring as it does the following steps: Homogenization, Decontamination, Centrifugation, Neutralization, Inoculation of the culture media and Incubation for upto 8weeks regularly examination of cultures (Collee *et al.,* 1999).

Inoculated culture media are usually incubated at 37˚C in atmosphere of 5% to 10% CO_2 for at least 8 weeks in slanted position with caps loose for at least one week to ensure even distribution of inoculum over the surface. *M. tuberculosis* appears within 4 weeks, but they may not be visible for 8 weeks or more if they originated from patients treated with anti-tuberculosis agents.

3.13.4 Identification tests

3.13.4.1 Rate of growth

Tubercle bacilli grow slowly compared to other non-pathogenic acidfast organisns. Based on the rate of growth, mycobacteria are grouped into slow growers and rapid growers. The slow growers take more than 7 days to appear on the culture media, whereas rapid growers will grow in less than 7 days.

3.13.4.2 Growth at 25°C (RT) and 42°C

The pathogenic mycobacteria like MTB and *M. bovis* do not grow at 25°C and 42°C.The optimum temperature for their growth is 37°C.The saprophytes grow well at RT and above. The atypical strains generally grow slowly at RT, and some grow at 42°C.All the AFB grows well at 37°C.Therefore, *M. tuberculosis* and *M. bovis* can be differentiated from others by their ability to grow at RT and at 42°C.

3.13.4.3 Growth in para-nitro benzoic acid medium (PNB medium)

Human and bovine type of tubercle bacilli can be differentiate from all other mycobacteria in their inability to grow in LJ medium containing 500mcgm/ml of PNB acid. Occasionally human strain may give some faint growth in this medium. The result of this and niacin test will help typing human and bovine strains.

3.13.4.4 Biochemical tests

3.13.4.4.1 Niacin test

This test is based on detection of the presence of nicotinic acid, and intermediate in the biosynthesis of NAD, in the culture medium. Niacin (Nicotinic acid) plays an important role in the oxidation-reduction reactions that occur during mycobacterial metabolism. Although all species produce nicotinic acid, *M. tuberculosis* accumulates the largest

amount. A positive niacin test is preliminary evidence that an organism that exhibits a buff-colored, slow growing rough colony may be *M. tuberculosis* (Forbes *et al*., 2002).In *M. tuberculosis*, this metabolic pathway blocks a large amount of nicotinic acid is excreted in the cultural medium. This test differentiates *M. tuberculosis* (99.5% species) from most other mycobacteria. The reagent contains 10% cyanogens bromide and 40% aniline in ethanol. When a freshly prepared reagent is added to a suspension of bacterial culture (grown in egg medium), a canary yellow colour shows a positive reaction.

INH (isoniazid) test strips are used for detecting isonicotinic acid (Niacin) and its metabolites in the aqueous extract of organism and medium. The INH test strips are absorbent strips impregnated with chloramines T, potassium thiocyanate, citric acid and barbituric acid. The pyridine ring of isonicotinic acid splits by cynogen chloride to form a glutacoaldehyde derivative. The derivative condenses with barbituric acid to form ablue purple polymethine dye.

For reliable results, the niacin test should be performed only from cultures on LJ that are at least 3 weeks old and show at least 50 colonies; other, enough niacin might not have been produced to be detected.

3.13.4.4.2 Nitrate test

In the Nitrate reduction test, the presence of nitrite (product of the Nitroreductase enzyme) is detected. *M. tuberculosis* suspended in a buffer solution containing nitrate and incubated at 37˚C for 2 hours reduces nitrate to nitrite which gives a pink or red colour when treated with sulphanilamide and N-naphthlyethylene diamine dihydrochloride.

The test is based on the principle that the enzyme nitrate reductase causes the reduction of the nitrate, in the presence of a suitable electron donar, to nitrite or nitrogen.

This test detects the ability of mycobacteria to reduce nitrate to nitrite. *M. tuberculosis* and *H37RV* strain are the nitrate reduction positive whereas *M. bovis* and *M. intracellutarae* are nitrate reduction negative.

3.13.4.4.3 Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase from non-catalase producing bacteria. Catalase is an intracellular, soluble enzyme capable of splitting H_2O_2 into water and oxygen. The oxygen bubbles into the reaction mixture indicating catalase activity. Catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Virtually, all possess catalase enzyme, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis.*

Reduced flavoprotein reacts directly with gaseous oxygen to form hydrogen peroxide which is an oxidative end product of the aerobic breakdown of sugars (Collee *et al.*, 1999).

$$FPH_2 + O_2 \longrightarrow FP + H_2O_2$$

Reduced flavoprotein — Oxidized flavoprotein

Hydrogen peroxide, thus formed, is toxic to bacteria, resulting in their death. So the enzyme catalase decomposes H_2O_2 into water and oxygen.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

3.13.4.5. Immunological test

Tuberculin test

The tuberculin test discovered by Von Pirquet in 1907.A positive reaction to the test accept as evidence of past or present infection by *M. tuberculosis* .The tuberculin test is the only means of estimating the prevalence of infection in a population.There are three main tests currently in use: the Mantoux Intradermal Test, the Heaf Test and the Tine Multiple Puncture Tests. The Heaf Test is usually preferred for testing large groups of people because it is quick and easy to perform, reliable and cheap. The Mantoux Intradermal Test favours when a more precise measurement of tuberculin sensitivity is required. The Tine Test is considered by some authorities as unreliable and therefore not recommended.

Tuberculin is a purified protein, derived from tubercle bacilli.Thus, another name for tuberculosis is PPD (Purified Protein Derivative).Following infection with, a person develops hypersensitivity to tuberculin protein. Tuberculin injected into the skin of an infected person produces a delayed local reaction after 24-48 hours. We quantify this reaction by measuring the diameter of the skin indurations (thickening) at the site of the reaction.

Result:

(i)A definite indurations with a diameter of 10mm and erythema indicate positive reaction =1+

(ii)Erythema and indurations 10 to 20mm =2+

(iii)Erythema and indurations more than 20mm =3+

(iv)Erythema and indurations with necrosis =4+

The reaction is negative, when the indurations is less than 6mm, or there is no indurations at all.Non-specific reaction usually fades away within 48 hours. Focal reaction characterized by exacerbation of the lesion and general reaction associates with malaise, fever, and other constitutional symptoms.

The usefulness of tuberculin is limited by its lack of specificity for tuberculosis and its inability to distinguish between active disease, prior sensitization by contact with *M. tuberculosis*, BCG vaccination, and cross-sensitization by other *Mycobacterium* species. The tuberculin test also fails to detect a substantial proportion of persons coinfected with human immunodeficiency virus (HIV) and *M. tuberculosis* and of person with advanced tuberculosis. Its sensitivity for active disease varies considerably from 65% to 94%.

Despite these limitations, the tuberculin skin is still very useful tool, especially when conversion to a positive skin test is used to identify recently infected persons for preventive therapy or to help confirm a physician's suspicion of tuberculosis.

3.13.4.6 Serological test

Serological tests for the diagnosis of tuberculosis based on the recognition of serum IgG antibody to selected mycobacterial antigens that use enzyme linked immunosorbent (ELISA) technique have been developed. They often lack both adequate sensitivity and specificity to be useful in clinical work.

Recently, a new simple to perform, 20 minutes immunoassay, the Myco-Dot antibody test has become available. It is a comb-dipstick, manufactured by Mossman Associates.Myco-Dot uses purified lipoarabinamannan as antigen (highly immunogenic lipopolysaccharide found in the cell wall of all mycobacteria).Myco-Dot has been design for diagnostic use,i.e. it detects anti-mycobacterial antibody levels likely to be found in those with active disease,i.e. high levels. Myco-Dot test should therefore only be performed on those with suspected tuberculosis (It is not a screening

test).A positive test indicates active tuberculosis. A negative Myco-Dot test, however, cannot exclude tuberculosis because a negative result may be due to a few antibody titre as occurs in immunosuppressant or circulating antigen-antibody complex lowering the antibody titre,or extra pulmonary tuberculosis in which the antibody level is below that which can be detected by the test (Cheesbrough, 2002).

3.13.4.7 New technology for diagnosing tuberculosis

Most of the recent advances in the laboratory diagnosis of tuberculosis have been directed at the development of rapid culture, identification and drug susceptibility systems for use in TB specialist laboratories.

3.13.4.7.1 Bactec 460 TB rapid radiometric culture systems

Developed by Becton Dickson Diagnostic system, the Bactec is an automated early detection system, in which specimens are cultured in a liquid media containing C^{14} labeled palmitic acid. Growing mycobacteria utilize the acid, releasing radioactive CO_2 which is measured in the BacTec instrument. Growth of *M. tuberculosis* can be detected within 12 days (WHO, I998).

3.13.4.7.2 Bactec 9000 MB system

This instrument is based on the continuous growth monitoring system. Organisms are cultured in a modified Middlebrook 7H9 broth. The instrument detects growth by monitoring O_2 consumption by mean of fluorescent sensor.

3.13.4.7.3 Septi-check AFB system

Developed by Becton Dickson, this system consist of biphasic culture system made up of a modified Middlebrook 7H9 broth with a three sided paddle containing chocolate,

egg- based, and modified 7H11 solid agars. The bottle is inverted regularly to inoculate the solid media. Growth is detected by observing the three-sided paddle.

3.13.4.7.4 Mycobacteria growth indicator tube (MGIT)

This system is developed by Becton Dickson. This consists of culture tube containing Middlebrook 7H9 broth and a fluorescent compound embedded in a silicon sensor. Growth is detected visually using an ultraviolet light. Oxygen diminishes the fluorescence output of the sensor; therefore, O_2 consumption by organism present in the medium is detected as an increase in fluorescence. This system provides easy to use system with high accuracy for easy detection of mycobacterial growth (medium 11 to 13 days) from clinical specimens.

3.13.4.7.5 ESP culture system II

This works continuous growth monitoring system. Organisms are cultured in a modified Middlebrook 7H9 broth with enrichment and a cellulose sponge to increase the culture's surface area. The instrument detects growth by monitoring pressure changes that occur as a result of O_2 consumption or gas production by the organisms as they grow (Forbes *et al*., 2000).

3.13.4.7.6 Polymerase chain reaction (PCR)

PCR, a well developed technique, is used extensively for the diagnosis of TB (Forbes and Hicks, 1993).It is a DNA based technology which produces relatively large number of copies of DNA molecules from minute quantities of source DNA from viable or nonviable cell material even when the source DNA is of relatively poor quality.PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension.

PCR enables the amplification of specific sequences of target nucleic acids. It is not only simple and fast, but also very sensitive and specific to amplify even a single molecule of DNA.

With the increased incidence of TB and the advent of MDR-TB strains, the demand of PCR is high in developing countries. The PCR-micro plate hybridization assay was also sensitive enough to detect as little as 1pg of DNA; which is equivalent to approximately three bacilli. Nowadays, PCR could become a valuable alternative approach to the diagnosis of TB infections. Recently, a commercial PCR amplification Kit for the detection and identification of *M. tuberculosis* complex bacteria has became available. The target for the PCR is the 16 S rRNA sequence. The detection system is based on hybridization with *M. tuberculosis* complex specific capture probe in a micro plate format.

3.13.4.7.7 Chromatographic analysis

The analysis of mycobacterial lipids by chromatographic methods, including thin layer chromatography (TLC), gas liquid chromatography (GLC), capillary gas chromatography methods and reverse-phase high performance liquid chromatography (HPLC) has been used to identify mycobacteria.HPLC of extracted mycobacteria was a very specific and rapid method for identification of species (Forbes *et al*., 2002) .

3.13.4.7.8 Bacteriophage-based test to detect *M. tuberculosis* in sputum

A new bacteriophage-based sensitive assay called FAST plaque TB has been developed by Biotec laboratory as an affordable test which can be performed in microbiology laboratories in developing countries. In the FAST plaque TB test, the patient's sputum mixes with reagent which contains mycobacteriophage (phage virus that infects *M. tuberculosis*). Following infection of *M. tuberculosis* by the phage, a virucid is added which destroys any mycobacteriophage that remains outside the tubercle bacilli leading to lysis of these cells and the release of phage. Rapid (overnight) growing non-

pathogenic mycobacteria which can also be infected by the phage are added and the sample is incorporated in an agar mixture, plated and incubated overnight at 37^0C. The phage replicates, infects and lyses the nonpathogenic mycobacteria, leaving of clearing (holes) in the agar. These areas of clearing indicate that the patient's sputum sample contained viable *M. tuberculosis*.

Biotec also supplies another new test called FAST plaque TB-RIF which identifies rifampicin resistant in *M. tuberculosis* strains within 48 hours from a culture. New tests are also being developed for drug susceptibility testing directly on smear positive sputum (Cheesbrough, 2002).

3.13.4.7.9 Antigen detection

Mycobacterial antigen is detectable in clinical specimens by one of specific antibodies in agglutination technique and Enzyme Linked Immunosorbent Assay (ELISA). Development of such test have been overshadowed by DNA technology but results of the few studies of their use with 'clean' specimens such as cerebrospinal, pleural and peritoneal fluids were increasing with high sensitivities and specificities.

3.13.4.7.10 Antibody detection

The detection of antibodies against mycobactainal antigens in sera from patients in ELISA has shown promising results. The most promising purified antigen in a number of ELISA is the 38KDa antigen from *M. tuberculosis*.The 10KDa, 16KDa and 24KDa proteins isolated from *M. tuberculosis* were also useful in.

3.13.4.7.11 ELISPOT test

Of the numerous immunological methods for diagnosis of TB, one promising test is ELISPOT test. It measures the antibody secreting cells in response to stimuli from a specific antigen. However, owing to its cost and requirements for special expertise, this test though useful, would not be suitable for use in a routine clinical laboratory.

3.13.4.7.12 Tuberculostearic acid (TBSA) test

One easily detected components *M. tuberculosis* is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography (Brooks *et al.,* 1991). The presence of tuberculostearic acid in cerebrospinal fluid is thought to be diagnostic for tuberculous meningitis and has been suggested to be useful in dig nosing pulmonary tuberculosis. However, an important concern with pulmonary specimens is that organisms other than *M. tuberculosis* may produce components that will generate a false positive signal (Bloom, 1994).

3.13.4.7.13 Molecular methods

In the field of infectious disease testing, molecular diagnostic method have been developed to replace conventional diagnostic methods that lacked sensitivity, specificity or were simply too slow. Molecular testing can yield genetic information about the virulence and antibiotic resistance of a particular microorganism (Bloom, 1994). By providing reliable and definitive identification, these methods would help in the patient management while monitoring drugs therapy and prophylactic measures (Sritharam and Sritharam, 2000).

Molecular methods relay on extraction, desired/ targeted nucleic acid amplification of conserved gene sequences of *M. tuberculosis*.These methods provide rapid detection, identification and characterization of *M. tuberculosis* strains. Different target sequences have been used to confirm the diagnosis of TB (Niemann *et al*., 2000).

Several molecular procedures useful for diagnosis of mycobactainal diseases include strand displacement amplification (SDA), polymerase chain reaction (PCR) amplification transcription-mediated amplification (TMA), reporter phase systems, oligonulcleotide ligation amplification and Q-beta replicase amplication.The first four of these amplification systems are the best developed of the systems for mycobacteria (Bloom, 1994).

3.13.4.7.14 Strand displacement amplification (SDA)

SDA is an isothermal amplification process that takes advantage of ability of DNA polymerase to start at the site of a single –stranded nick in double-stranded DNA,extend one strand from the 3' end, and displace the downstream strand of DNA (Walker,2001) .The replicated DNA and the displaced strands are then substrates for additional round of oligonucleotide annealing,nicking,and strand displacement such that the amplification proceed in a geometric manner and can produce 10^7 to 10^8 fold amplification in about 2 hours(Bloom,1994).The specificity of the SDA reaction is based on the choice of primers to direct the DNA synthesis. When coupled with chemiluminescence-based hybridization detection system, the entire assay can be completed within 4hours of obtaining a processed specimen. Species specific SDA assays have been developed for *M. tuberculosis*, *M. avium* and *M. kansaii*. An assay that detects many members of *Mycobacterium* genus (a genus specific assay) has also been developed (Bloom, 1994).

3.13.4.7.15 Transcription-mediated Amplification (TMA)

TMA, an isothermal target based amplification system developed by Gen-Probe Incorporation, has been combined with a homogeneous detection method to detect *M. tuberculosis* in clinical specimens (Jonas *et al.*, 1993) and rRNA is amplified via TMA in which the rRNA target sequences are copied into a transcription complex by using reverse transcriptase and then RNA polymerase is used to make numerous RNA transcripts of the target sequence from the transcription complex. The process then repeats automatically. Detection of the amplified sequence is achieved by using 'an acridium ester-labeled DNA probe specific for *M. tuberculosis* (Bloom, 1994).

3.13.4.7.16 Reporter mycobacteriophage

A reporter mycobacteriophage is a virus that infects the desired *M. tuberculosis* and produces an easily measured product. The specificity of this approach lies in the host

range specificity of the reporter phage. There are phages that can infect only *M. tuberculosis* as well as ones that can grow in several species of *Mycobacterium*.The sensitivity of the system lies in the synthesis of large amount of the reporter product during phage growth(i.e. amplification of the product) and in the sensitivity of the assay in detecting the reporter product. Jacobs *et al.*(1993) recently constructed a reporter phage for detecting *M .tuberculosis* that carries the gene for the firefly enzyme luciferase.In the presence of ATP,this enzyme oxidizes luciferin to generate light, which is the reaction that makes firefly glow in the dark.

3.14 Treatment

The development of effective treatment for tuberculosis has been one of the most significant advances during this century. The modern treatment of the tuberculosis based on the administration of effective drugs. In the presence of adequate chemotherapy, hospitalization, rest and improved diet do contribute to achieving cures. Tuberculosis is a curable disease and treated with oral drugs sometimes together with injection. TB drugs are available at free of cost in government health facilities in Nepal. The total duration of treatment is 6 to 8 months. Treatment should not be discontinued before completion of full course; the drug resistance will develop which is dangerous to patients as well as to community. Drug resistance TB is difficult to treat (STC,2003).So in order to prevent the emergence of drug resistant mutants which are present in very small numbers, at least to effective drugs are always required. On account of long generation time of mycobacteria and their long periods of metabolic inactivity, prolonged courses of drug therapy are always recommended.

The objectives of the anti-TB drugs treatment (WHO, 1997):

i. To cure the patients of TB

ii. To prevent death from active TB or its late effects

iii. To prevent TB relapse

iv. To decrease TB transmission to others

There are now twelve or thirteen drugs active against *M. tuberculosis* of which five are considered to be essential. One anti-TB drug should satisfy the following criteria:

a. Highly effective

b. Free from side effects

c. Easy to administer and

d. Reasonably cheap

The currently used drugs may be classified into two groups: bactericidal and bacteristatic. The bactericidal drugs kill the bacilli in vivo while bacteristatic drugs inhibit the multiplication of the bacilli and lead to their destruction by the immune mechanism of the host (Park, 2000).

Traditionally, antituberculous drugs have been classified as first line drugs having superior efficacy with acceptable toxicity. These are Isoniazid (H), Rifampicin (R), Pyrazinamide (Z), Streptomycin (S) and Ethambutol (E). The second line drugs either having less efficacy, greater toxicity or both are Thiacetazone, Cycloserine, Kanamycine, Amikacin, Capreomycin, Viomycin, Para-aminosalicylic acid.

Five drugs regarded as essential in the treatment of tuberculosis are isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol. Thiacetazone is also used to supplement isoniazid in many developing countries because of its low cost. (WHO, 1993). Anti-TB drugs have three main properties – bactericidal ability, sterilizing ability and the ability to prevent resistance. The essential anti-TB drugs possesses these

properties to different extents. Isoniazid and Rifampicin are the most powerful bactericidal drugs, active against all population of TB bacilli. Rifampicin is the most potent sterilizing drug available. Pyrazinamide and streptomycin are also bactericidal against certain populations of TB bacilli. Pyrazinamide is active in an acid environment against TB bacilli inside macrophages. Streptomycin is bactericidal against rapidly multiplying TB bacilli. Ethambutol and thiacetazone are used in association with more powerful drugs to prevent the emergence of resistant bacilli (WHO, 2003).

TB treatment regimens divide in to the initial or intensive phase and the continuation phase. The intensive phase means daily treatment with a combination of 4 drugs-INH, RMP, PZ and EM, s or S-for two months. During the initial phase, there is rapid killing of TB bacilli. Infectious patients become non-infectious within 2 weeks and symptoms improve. The vast majority of patients with sputum smear-positive TB become sputum smear-negative within 2 months. Directly Observed Therapy (DOT) is essential in the initial phase to ensure that the patient takes every single dose. This protects Rifampicin against the development of drug resistances. The risk of drug resistance is higher during early stages of anti-TB drug treatment when there are more TB-bacilli. The continuation phase means daily treatment with Isoniazid (INH) and Rifampicin (RMP) for six months. The drugs eliminate the remaining TB bacilli, killing the persisters, prevent relapse after completion of treatment. DOT is the ideal when the patient receives Rifampicin in the continuation phase. The risk of resistance is less during the continuation phase when there are fewer TB bacilli (NTP, 1998).

DOTS, a TB control strategy, pioneered by International Union against TB and Lung Disease and recommended by WHO to ensure cure by providing the most effective medicine and confirming that it is documented to be effective world-wide on a programme basis. In DOTS, during the intensive phase of treatment, a health worker or other trained person watches as the patients swallow the drug in his presence. During continuation phase, the patient is issued medicine for one week in a multiblister combipack of which the first dose is swallowed by the patient in the presence of health

worker or trained person. The consumption of medicine in the continuation phase is also checked by return of empty mutliblister combipack when the patient comes to collect medicine for the next week. DOTS was introduced in Nepal in 1996, and successfully implemented throughout the country since. April 2001. By July 2002 DOTS had been expanded to 273 treatment centers with 909 subcentres all over the country with 89% of population coverage by DOTS. 89% is the success rate of DOTS in Nepal DOTS in now (July 2005) running through the integrated general health services in 462 treatment centres and 2482 subcentres throughout Nepal. Now almost all diagnosed TB patients are getting treatment under DOTS strategy with more than 85% treatment success rate. The high treatment success rate of new smear positive cases has been sustained for the very beginning. The defaulter rate is declining now i.e. 3.6% in 2005 vs 5% in the last fiscal year. According to Annual Report 2003/2004, NTP, Nepal, the country has expended the DOTS strategy to all the districts by July 2001. Now in the process of expansion of DOTS in each and every health institution. The Programme has achieved the TB control targets of case detection and treatment success, as of July 2004 the case detection rate was 71%. The cure rate and the success rate for the previous years (2003/2004) were 86% and 87% respectively.

3.14.1 Treatment categories

Treatment regimens contain combined drugs to prevent the emergence of resistant strains. There are many different possible anti-tuberculosis treatment regimens. The WHO and the International Union against Tuberculosis and Lung Disease (IUATLD) recommended standardized tuberculosis treatment regimens.

3.14.1.1 Treatment categories in Nepal (NTP)

Patients are categorized according to their priority for treatment (highest to lowest). In general, newly diagnosed cases and those with smear positive pulmonary tuberculosis

and other clinically serious forms of diseases should be accorded the highest priority. The National Tuberculosis Programme (NTP) of Nepal has adopted 3 categories for treatment of TB as recommended by WHO (WHO, 1993).

A. **Category I**

2HRZE/6HE i.e. this category contains new cases of smear positive pulmonary tuberculosis, new smear negative PTB with extensive parenchyma involvement and other newly diagnosed seriously ill patients with several forms of extra pulmonary tuberculosis.

Recommended regimens

Intensive phase

2HRZE (S) i.e. isoniazid, rifampicin, pyrazinamide and either ethambutol or streptomycin given daily for 2 months. Streptomycin replaces by Ethambutol in the intensive phase because of increased prevalence of HIV in this region.

When the patient completes the initial phase of treatment and the sputum smear negative, the continuation phase starts. However, if the sputum is smear positive at 2 months, the initial phase of therapy should be extended by 4 weeks. The patient should then start the continuation phase, regardless of the results of the sputum test.

Continuation phase

6HE i.e. isoniazid and ethambutol, given daily for 6 months. For patients with tuberculous meningitidis, disseminated tuberculosis or spinal disease with neurological complications, isoniazid and rifampicin should be given daily for 6-7 months (i.e. a total and 8 months and therapy).

Alternative continuation phase

6HE (T) i.e. Isoniazid and Ethambutol or Isoniazd and Thioacetazone, given daily for 6 months.

B. Category II

2HRZES/IHRZE/5HER i.e. these are the patients who have received anti-tuberculosis treatment for more than one month in the past. They are therefore at an increased risk of having multi-drug resistant disease. These include smear-positive relapses, smear positive failure cases and smear-positive patients being treated after default.

Recommended regimen

Intensive phase

2HRZES/IHRZE i.e. rifampicin, isoniazid, pyrazinamide and ethambutol, given daily for 3 months and supplemented with streptomycin for the first 2 months .When patient has completed the initial phase of treatment and the sputum is smear-negative, the continuation phase is started. However, if the sputum is smear positive at 3 months, the initial phase of treatment with the four drugs should be stopped for 2-3 days and a sputum specimen sent to the laboratory for culture and susceptibility testing. The patient should start the continuation phase.

If the pre-treatment studies showed resistance to Isoniazid or Rifampicin alone, the patient should start the continuation phase under close supervision. In this case sputum conversion is likely to be achieved, provided that all doses of drugs taken until the end of the treatment.

If the pre-treatment studies showed resistance to both Isoniazid and Rifampicin or resistant to both these drugs is found in a patient who remain smear positive at the end of the initial phase, the chance of achieving sputum conversion is limited.

Continuation phase

6HER i.e. Isoniazid, Rifampicin and Ethambutol for 6 months. If the patient remain smear positive after the completion of the continuation phase, he or she has no longer for the retreatment regimen.

C. Category III

2HRZ/6HE i.e. this includes the patients with pulmonary smear negative tuberculosis with limited parenchymal involvement and extra-pulmonary tuberculosis.

Recommended regimens

Intensive phase

2HRZ i.e. Isoniazid, Rifampicin and Pyrazinamide given daily for 2 months.

Continuation phase

6HE (T) i.e. Isoniazid and Ethambutol or Thioacetazone given daily for 6 months.

D.Category IV

This category includes the patients with chronic tuberculosis (still sputum positive after supervised retreatment).Low management of these patients, who have high likelihood on MDR-TB, highly problematic. Even with optimal therapy, cure may be possible in only half of such cases. Second line drugs are used for these patients.

3.14.1.2 Registration categories of TB patients

After diagnosis, the patient must be registered and should be properly treated. The registration categories of TB patients will help the health workers to decide which

category of treatment is appropriate to the patient. Most importantly, in order to identify those patients at increased risk of acquired drug resistance and to prescribe appropriate treatment, a case should be defined according to whether essential epidemiological monitoring of the TB epidemic at regional and country level. The following definitions are used.

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

Relapse: A patient previously treated for TB and who has been declared cured or treatment completed, and is diagnosed with bacteriological positive (smear or culture) tuberculosis.

Treatment after failure: A patient who while on treatment, remained smear positive or once more become smear positive or smear positive at the 5^{th} month or later during the course of treatment or was initially smear-negative before starting treatment and become smear positive after the 2^{nd} month of treatment.

Return after default: A patient who completed at last 1 month of treatment and returned after at least 2 months interruption of treatment.

Transfer in: A TB patient already registered for treatment in one district that transfers to another district where he/she continues treatment. The patient must have been taking anti TB drugs for up to the end of 2 months.

Chronic: A patient who reminded smear sputum positive after completing a directly observed re-treatment regimen (WHO, 2003).

Follow up: A TB patient whose sputum specimens are examined during the treatment for checking the effectiveness of the treatment; the patient must have the sputum examination after 2(3*) months, 5 months and in the last months of treatment.

- For category 2 patients.

Others

Case who doesn't fulfill the above criteria. This is usually a patient who has been taking anti-TB drugs for more than 4 weeks but has not been registered within the NTP. Most of these will be patients who come into the NTP for their treatment from hospital or private practioners.

3.15 Adverse effects of anti-TB drugs

Most TB patients complete this treatment without any significant adverse effects of drugs. However, a few patients develop adverse effects. Adverse effects are classified as minor or major. In general, a patient who develops minor adverse effects should continue the anti-TB treatment, usually at the same dose but sometimes at a reduced dose. The patients also receive symptomatic treatment. If a patient develops a major side effect, the treatment or the offending drug is stopped.

It has been suggested that only a minority of patients successfully complete their full course of anti-tuberculosis (TB) chemotherapy without significant side-effects. There is also an opposing view that most patients with TB complete their treatment without serious adverse effects. What is the truth? Modern anti-TB chemotherapy regimens have been in use for >30 years. However, the frequency of severe complications is not well known, probably due to lack of notification and under-reporting. It is clear that many patients have adverse reactions which complicate treatment and have an influence on treatment outcomes. However, it is difficult to measure the efficacy or toxicity of a particular drug, since anti-TB drugs are usually administered in combination regimens of several drugs. Therefore, any care provider treating a TB patient is assuming a public health function that includes not only prescribing an appropriate regimen, but also ensuring adherence to the regimen and monitoring of the treatment, including the side-effects of drugs until treatment is completed.

There is still much debate concerning the frequency and severity of symptoms of TB when undergoing chemotherapy. In addition, the frequency of complications is hard to quantify as many patients are treated with a range of different drugs. However, this article aims to give a brief overview of the side-effects associated with anti-TB drugs and the most appropriate management approaches to take.

The main adverse effects of anti-TB drugs usually occur during the first 2–3 weeks of treatment. If these side-effects are not recognized on time and managed properly they can lead to treatment interruption or can even can be life threatening. Proper monitoring has to be carried out during the whole treatment course, including patient education, clinical examination, laboratory tests, *etc*.

If symptoms of adverse effects occur the following should be done:

- the dose of drugs should be checked
- all other causes of symptoms should be excluded
- the seriousness of the adverse effects should be estimated
- the adverse effects should be registered
- the drugs should eventually be reintroduced gradually when symptoms disappear
- the development of drug resistance should be avoided

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		Stop responsible drugs
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, acute renal failure	Rifampicin	Stop rifampicin

Table 2. Symptom based approach to adverse effect of TB drugs (WHO, 1996)

3.16 Development of drug resistant tuberculosis

Two great problems have dogged the successful drug therapy of TB since its inception. The first of these has been the organizational and financial difficulties in ensuring that all patients receive a complete course of the most effective drugs currently available. The second problem has been that of the emergence of bacilli resistant to the drugs (Yates *et al.*, 1985). The spectrum of drug resistance is becoming a more credible challenge in many pasts of the world dimming the prospects of eventual elimination (Lauzardo and Ashken, 2000).

Drug resistant tuberculosis is a case of tuberculosis (usually pulmonary) excreting bacilli resistant to one or more anti-tuberculosis drugs. Resistance of *M. tuberculosis* to anti-tuberculosis drugs is a man-made amplification of natural phenomenon (Rijal *et al*., 2002).

The prevalence of drug resistance varies geographically and is generally higher in developing countries than in technically advanced countries. Drug resistance is a natural phenomenon in *M. tuberculosis* due to random mutations which occur in 10-6 to 10-9 bacillary replications (Viskum and Kok, 1997).

The failure of TB control programmes and drugs resistance encouraged the world Health Organization (WHO) to propose strength National Tuberculosis control programme to treat successfully 85%of sputum positive detected cases through introduction and improved application of short causes chemotherapy, and to establish a global drug resistance surveillance system (Shrestha *et al.*, 1996).

Modern anti-tuberculosis drug cures most tuberculosis cases 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). Multi Drug Resistant (MDR) tuberculosis is resistant to at least isoniazid and rifampicin, the main anti-tuberculosis drugs (Rijal *et al.*, 2002). It has been observed that increasing unsupervised and improper use of anti-tuberculosis drugs lead to resistance (Malla, 1996). Resistance may be due to problems in drug supply, inappropriate prescribing, poor patient compliance, inadequate TB programme

support or supervision, substandard drug quality and malabsorption (Warndorff *et al.*, 2000).

The emergence and spread of MDR-TB threat global TB control (Mendez, *et al.*, 2002). The susceptibility patterns of *M. tuberculosis* isolates against anti-tuberculosis drugs informs an important aspect of TB controls 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.

3.17 Types of drug resistance

Drug resistance means that certain strains of tuberculosis bacilli are not killed by anti-tuberculosis drugs given during the treatment. All drugs used in the treatment of tuberculosis tend to produce resistance strains (Park, 2000). There are two types of resistance .They are as follows:

3.17.1 Primary or pretreatment resistance

It is the resistance shown by the bacteria in a patient who has not received the drug in question before. In other words, it occurs in patients who have not had prior treatment with anti-tuberculosis drugs. This type of resistance is observed when a patient develops TB after being infected by another patient who has resistant TB organisms.

3.17.2 Acquired or secondary resistance

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 become resistant to the

particular drug during the course of treatment with it (Park, 2000). It occurs in patients with some record of previous drug treatment (WHO, 2002). This is mostly because of single drug-due to irregular drug supply, in appropriate prescription or poor adherence to treatment suppress the growth of the susceptible bacilli to that drug but permits the multiplication of drug resistant organisms (Rijal *et al.*, 2002).

Factors associated with anti-tuberculosis drug resistance:

1. Programmatic factors

* Lack of a standardized therapeutic regimen

* Poor implementation compounded by frequent or prolonged shortage of drug supply areas with inadequateness occurs

* Political instability

* Use of anti- tuberculosis drugs of unproven quality

2. Health provided related factors

* Improper or inappropriate management of individual cases

* Selection of inappropriate regimen due to lack of recognition of prior treatment

* Ignorance of importance of standardizes regimens

* Addition of single drug to a failing regimen

* Lack of proper monitoring or supervision patients while on therapy

3. Patient related

*Non-adherence to prescribed treatment

*HIV infection

3.18 Multi drug resistant tuberculosis (MDR TB)

Multi drug resistant tuberculosis (MDR-TB) is defined as a form of tuberculosis caused by strains of *M. tuberculosis* that are resistant to at least isoniazid (INH) and rifampicin (RMP) .Resistant against the two most potent drugs may interfere with the future control of TB and complete cure may not be achieved due to lack of efficient drugs for treatment. In particular, treatment of patients with multi drug resistance is longer, costly and requires the use of drugs which frequently causes severe achieves reactions (Iseman, 1993). MDR is the most severe from of bacterial resistance today. It is why MDR tuberculosis is an important concern for tuberculosis control in many countries.

Since the early 1990s, several outbreaks of MDR tuberculosis have been reported in different regions of the world, as a consequence of in appropriate case of essential anti-tuberculosis drugs. Usually MDR tuberculosis occurs in chronic cases or other pretreatment regimens and represents significant properties on of tuberculosis patients with acquired with resistance. Exceptionally, MDR tuberculosis drugs, and who have been infected by MDR bacilli. In most settings, these new cases with MDR bacilli represent a very small proportion of new tuberculosis patients with primary resistance (WHO, 1998).

Generally the bases for the management of MDR tuberculosis are as follows:

1. Designing an appropriate regimen

2. Reliable anti-tuberculosis drug resistance surveillance system.

3. Quality assurance of the susceptibility test caused out in any laboratory

4. Reliable anti-tubercular drug supplies

Factors contributing to development of MDR-TB

a. Clinical factors

* Unreliable treatment regimens

* Inadequate dosage/duration of drugs

* Addition of single drug to a failing regimen

* Free availability of anti-tuberculosis drugs over the country

* Interference by occult quack medicine

* Delay in diagnosis

b. Biological factors

* Genetic predisposition

* Large bacillary population

* Cavitary lesion, local factors inside the host favorable for the multiplication of drug resistant bacilli

* Presence of drug the insufficient concentrations in tissues

c. Social factors

* Failure of public health system.

* Neglect of disease

* Irregular or in adequate intake of drugs

* Poverty

d. Epidemiological risk factor for drug resistance

* Length of premium chemotherapy

* Living on area with high prevalence of drug resistant TB

* HIV infection

* Social instability

e. Pharmaceutical factors

* Insufficient concentration of pure drug

* Inadequate standardization of bio-availability of the drugs

* Improper storage condition of the drugs (Thakker and Shah, 1998; Malla, 1996)

3.18.1 Global Situation of DR-TB/MDR-TB

The World Health Organization (WHO) has estimated that up to 50 million people may be infected with resistant *M. tuberculosis* globally (WHO, 1998). Despite the advancement of effective chemotherapy to treat tuberculosis, disturbing trends are emerging affecting the curability of TB. One of which is certainly the rising prevalence of drug resistant strains of *M. tuberculosis* to anti-tuberculosis drugs primary as well as acquired.

Drug-resistant tuberculosis has been reported since the early days of the introduction of chemotherapy. However, most of the evidence was limited to developed countries. In 1992, the third world congress on tuberculosis concluded that there was little recent information on the global magnitude of multi-drug resistant tuberculosis (Espinal, 2003).

Increasing cases of MDR-TB caused morbidity and mortality world wide. To evaluate the magnitude of the problem of resistance of anti-tuberculosis drug world wide in 1994, the global Tuberculosis Programme of the WHO and the International Union Against Tuberculosis and Lung. Disease (IUATLD) initiated the Global Surveillance (Lee, 2001). The drug resistance surveillance of WHO/ IUATLD Global project shows that, globally MDR-TB cases were 1% in 64 countries. However, MDR-TB is at critical levels in specific regions of the world. Hot spots for MDR-TB include Estonia, Latvia, Oblasts of Invanovo and Tomsk in Russia, and the provinces of Henan of Zhejiong provinces in China. Two thirds of the world's countries and more importantly half of the22 tuberculosis high burden countries have not yet provided data. Mathematical modeling suggests that 3.2% (2,73000) of the world estimated new tuberculosis case were MDR-TB in 2000 (Espinal, 2003).

The first global report on DRS was released in 1997 and included date from countries/geographical sites. This report showed that drug resistance was ubiquitous. Median prevalence of resistance to at least on drug among new tuberculosis cases was 9.9% (range 2-41%) and MDR-TB was 1.4% (range 0-14%) and median prevalence of resistance to at least one drug among treated tuberculosis cases was 36% (range 5-100%) and MDR-TB was 13% (range 0-54%) (WHO, 1997).

A second global report followed in 2000 and included data from 58 countries/ geographical sites showed the prevalence of resistance to at last one drug among new cases was 10.7% (range 0-14%). Among previously treated cases, the prevalence of

resistance to at least one drug was 23% (range 0-94%) and MDR-TB was 9% (range 0-48%) (WHO, 2000).

The global burden of TB in SAARC region was 38% among these, in India, the data revealed that 13.4% of the new of old cases of MDR-TB in 1994-1997, according to WHO/UATLD. The recent surveillance, 2000 report of WHO/IUATLD showed that 3.4%of the new cases and 25% of the old cases has MDR tuberculosis in India (Rijal *et al.*, 2002). The main reasons for emergence of drug resistant in India were erratic drug ingestion, immunotherapy. Omission of one or more prescribed agents, suboptimal dose and poor drug absorption (Thakker and Shah, 1998).

3.18.2 Situation of DR/MDR-TB in Nepal

The extent of problem of drug resistance was not known in Nepal. Most of the studies carried out in KTM valley only but since last 6 years drug resistance surveillance of TB cases of Nepal carried out with the cooperation of NTC and National Anti-Tuberculosis Association (NATA)/German-Nepal Tuberculosis Project (GENETUP) Laboratory.

The initial drug resistance rate in Nepal is not so high comparing with that of other countries. In 1992, the primary drug resistance rate was 6.3%, 9.5% for INH and SM respectively. First surveillance of TB drug resistance survey which showed initial MDR 3.7% and acquired 12% (Shrestha,2002).Gharti Chettri,2002, reported that 8.57% of the isolates had primary MDR-TB while 100% of the isolates had acquired MDR-TB.Bhatteria,2003,reported 4.61 % and 5% of the total isolates had primary and acquired MDR-TB respectively.

The anti-tuberculosis drug sensitivity test conducted in Nepal during 1987-1990 revealed that 5.7% and 30% of new and old cases respectively were having MDR-TB and another study during 1991-1994 revealed that 1.6% and 9.6% of new and old cases respectively had MDR tuberculosis. The last surveillance report of the IUATLD/WHO, 2000 showed that 1% and 7.4% of new and old cases had MDR-TB in Nepal. MDR-TB

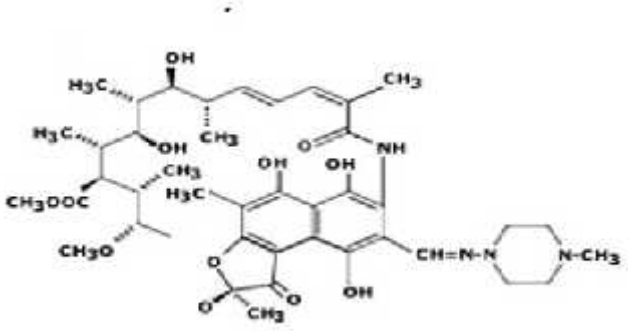
in new cases is reducing from 3.7% in 1998-1999 to 1.33% in 2001-2002 due to the DOTS programme (NTC, 2002). The latest surveillance report of the IUATLD/WHO, 2004 showed that 1.3% and 20%of untreated (new) and treated (old) cases had MDR-TB in Nepal.

3.19 Molecular basis of anti-TB drug resistance in TB

M. tuberculosis of ten acquired early in life with active infection and with developing immunity, granuloma formation and calcification. This is followed by along latest period, which continuous until reactivation occurs in a proportion of the individuals. This means that individual strains of *M. tuberculosis* have little opportunity to interact and exchange genetic information with other strains compared with, for example organisms that colonize the naso pharynx or the gastrointestinal tract. In these locations, other bacteria transmit antibiotic resistance determinants through transmissible genetic elements, transposons, integrons, and plasmids, by transduction or transformation. This option is not available for *M. tuberculosis*, so resistance can only occur through chromosomal mutation, although rarely movement of mobile genetic element, such as the insertion sequence IS6110 has been associated with new resistance emerging through the inactivation of critical genes.

The rate at which resistance emerges differs for all of the anti-tuberculosis agents, being highest for ethambutol and lowest for rifampicin. The rate of mutation for most of the anti-tuberculosis used in tuberculosis treatment were $3.32x10^{-19}$,$2.56x10^{-8}$,$2.29x10^{-8}$, and $1.0x10^{-7}$ mutations per bacterium per cell division for rifampicin, isoniazid, streptomycin and ethambutol respectively (Gillespie,2002).

3.19.1 Rifampicin resistance



$C_{43}H_{58}N_4O_{12}$ Mol. Wt. 822.9

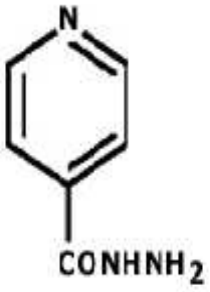
Rifampicin is (12Z,14E,24E) - (2S,16S,17S,18R,19R,20R, 21S,22R,23S) - 1,2-dihydro-5,6,9,17,19-pentahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-(4-methyl-piperazin-1-yliminomethyl)-1,11-dioxo-2,7- (epoxypentadeca - 1, 11, 13-trienimino) naphtha [2,1-b] furan- 21-yl acetate. (Indian Pharmacopoeia, 1996)

Rifampicin is the first line TB drug. This drug has highly effective bactericidal action against *M. tuberculosis* and makes a key component of the initial anti-tuberculosis regimen. Analysis of approximately 500 Rifampicin strains from global sources has found that 96% of Rifampicin resistant clinical isolates of *M. tuberculosis* have mutations in the 81-bp core region of rpoB gene, which encodes the B subunit of RNA polymerase. These mutations are absent in susceptible organisms. Although minor discrepancies have been reported, in general, their have been a strong correlation of a specific amino acid substitutions and MIC. Missense mutation in codon 513, 526, or 531 result in high level Rifampicin resistance, whereas amino acid change at position

514 or 533 usually result in low levels of Rifampicin resistance. It is estimated that 90% of rifampicin-resistant isolates in some areas are also resistant to isoniazid, making rifampicin resistance a useful surrogate marker for multi drug resistance and indicating that second and third the drugs to which these isolates are susceptible are urgently required.

Resistance of rifampicin arises due to mutation in a beta subunit of RNA polymerase encoded by the gene rpoB, inhibiting RNA synthesis. Missense mutation in RNA polymerase and alternation in cell wall permeability is responsible for rifampicin resistance (Gillespie, 2002).

3.19.2 Isoniazid resistance



$C_6H_7N_3O$ Mol. Wt. 137.14

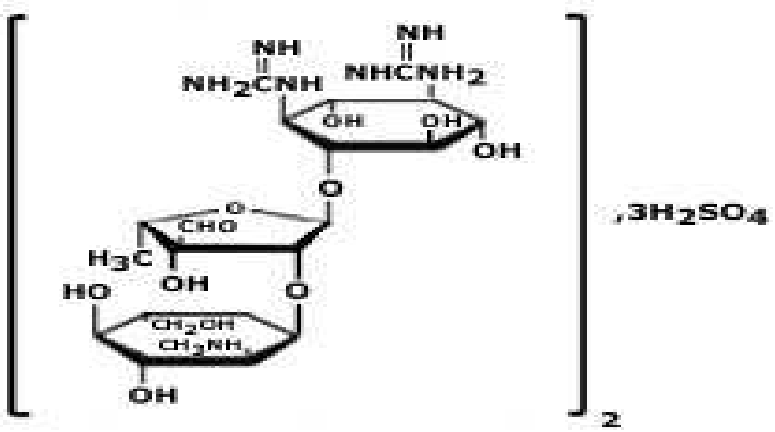
Isoniazid is isonicotinohydrazide. (Indian Pharmacopoeia, 1996)

Isoniazid is a synthetic, bacterial agent, used as the first line TB drug. Despite its widespread application to tuberculosis therapy and prophylaxes and intensive lab investigation, there is much that is not yet understood about the bacteria targets and mode of action of INH. Investigations on several continents have reported that many

(50-60%) INH resistant patient isolates have mutations, small deletions or insertions that are not represented among INH sensitive control strains. Mutations leading to INH resistance have been identified in different gene targets including Katg G, inha, ahpc and other genes that remain to be established. Telent *et al*. analyzed *M. tuberculosis* isolates by PCR and found that the mutation frequencies were as follows for INH resistant strains Katg G (36.8%), inhA (3/6%), Kat G-inhA (2.6%), ahpC (13.2%) and KatG-ahPC (2.6%). Amino acid replacements in the NADH binding site of InhA apparently result in INH resistance by preventing the inhibition of mycolic acid biosynthesis. Mutations in the KatG or inhA do not account for all INH resistant strains since 15-25% INH resistance clinical isolates have both wild-type KatG and inhA genes. The mechanism of INH resistance in some strains remains to be determined.

Scientists from Albert Einstein Institute of medicine, New York recently discovered a gene named inhA in *M. tuberculosis* which directs the production of enzyme responsible for the formation of bacterial cell wall. INH acts by binding the gene, there by preventing the production of enzyme and interfering with the lipids to kill the bacteria. Resistant to INH could occur either through or missense mutation that blocks INH from binding to the gene or through formation of large amounts of enzyme which could nullify the effect of INH. Such missense mutation in NHA import resistant to INH. Zhyac Young (1993) demonstrated another gene KatG, which encodes for the catalase peroxides reaction, for INH resistance (Thakker and Shah, 1998). The chance of spontaneous mutations occurring resulting in simultaneous resistance to INH and RMP was exceedingly small.

3.19.3 Streptomycin resistance



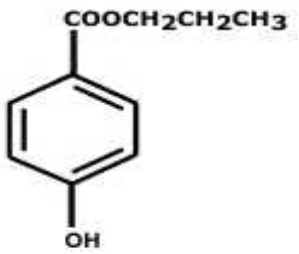
$(C_{21}H_{39}N_7O_{12})2,3H_2SO_4$ Mol. Wt. 1457.38

Streptomycin Sulphate is the sulphate of O-2-deoxy-2- methylamino-a -L-glucopyranosyl-(1-->2)-O-5-deoxy-3-C-formyl-a-L-lyxofuranosyl-(1->4)-NM^3-diamidino-D- formyl-a-L-lyxofuranosyl-(1-->4) -N1, N3-diamidino- D- (amendment 4) streptamine, a substance produced by the growth of certain strains of Streptomyces griseus or obtained by any other means. (Indian Pharmacopoeia, 1996)

Streptomycin inhibits protein synthesis in bacteria by binding to specific ribosomal protein in 30 Sribosomal subunit, Streptomycin resistance emerges through mutations in rrs and rpsl genes that produce alternation in the streptomycin binding site (Gillespie, 2002).Streptomycin is another first line TB drug, mutations associated with streptomycin resistance in tuberculosis have been identified in the 16 S rRNA gene (rrs) and rpsl gene. In to other bacteria that have multiple copies of rRNA gene, *M. tuberculosis* complex members have only one copy. Therefore, single nucleoside changes can potentially produced anti-biotic resistance. Mutation is the rrs are clustered in two regions around nucleotides 530 and 951. The 530 loop 16 S rRNA is highly conserved and is located adjacent to the 915 region in the secondary structure models. The majority of mutations producing streptomycin resistance occur in rPSL. The most common mutation is at the codon 43. Mutations also have occurred in codon 88. About

65.75% of Streptomycin resistant isolates have resistance-associated changes in rPSL or rrs.S. Failure to identify resistance-associated variations in these genes in 25-35% of organisms indicates that other molecular mechanism of Streptomycin resistance exists.

3.19.4 Pyrazinamide (PZA) resistance



$C_5H_5N_3O$ Mol. Wt. 123.11

Pyrazinamide is pyrazine-2-carboxamide. (Indian Pharmacopoeia, 1996)

Most Pyrazinamide resistant organisms have mutation in the Pyrazinamidase gene, although the gene are also be inactivate through the insertion of IS6110. Pyrazinamide produces the active pyrazinoic acid derivative, and mutations are unable to produce an active drug (Gillespie, 2002).

PZA is a structural analogue of nicotinamide that is used as a first line TB drug. PZA kills semi-dormant tubercle bacilli under acidic conditions. It is believed that in the acidic environment of phagolysomes the tubercle bacilli produce pyrazinamidase, an enzyme that converts PZA to pyrazinoic acid, the active derivative of this compound. To define the molecular mechanism of PZA resistance the *M. tuberculosis* pnc A gene encoding pyrazinamidase has been sequenced. The results have provided evidence that pncA mutations conferred PZA resistance. DNA sequencing of PZA resistance clinical isolates identified mutation at codon 63,138,141, and 162. In contrast, susceptible

organisms had wild type sequences. Lack of pncA mutation in 28% of PZA resistant isolates suggested the existence of at least one additional gene participating in resistance. A remarkably wide array of pncA mutations resulting in structural changes in the pncA has been identified in greater than 70% of drug resistant isolates. It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form.

3.19.5 Ethambutol resistance

$C_{10}H_{24}N_2O_2$, 2HCl Mol. Wt. 277.23

Ethambutol Hydrochloride is (S,S)-N,N `-ethylenebis (2- aminobutan– 1- ol) dihydrochloride. (Indian Pharmacopoeia, 1996)

Ethambutol is a bactericidal first line TB drug. This agent has been proposed to be an arabinose analog; the specific target is likely to be an arabinosyl transferase, presumably a functionally important site. To understand the mechanism of resistance of Ethambutol, a two gene locus (embAB) that encodes arabinosyl transfer has been established. Automated sequencing of there regions in clinical isolates from diverse geographical areas, discovered that 69% of Ethambutol resistance isolates had an amino acid substitution in EmbB that was not found in Ethambutol susceptible strains. The great majority (98%) of strains had mutation in codon 306; however, mutations were

also identified in three additional codons 285, 330, and 630. These mutations were also uniquely represented among Ethambutol resistance organisms. The data are consistent with the idea that specific amino acid substitutions in EmbB detrimentally affect the interaction between. Ethambutol, a putative arabinose analogue and Emb B likely to be an arabinosyl transferase. Emb B mutations are associated with Ethambutol resistance in roughly 70% of Ethambutol isolates of *M. tuberculosis.* The cause of Ethambutol resistance in many organisms lacking mutations in ERDR of Emb B is unknown (Mendez, 2002).

CHAPTER 4

4. MATERIAL AND METHODS

4.1 Materials

A complete list of bacteriological media, reagents, chemicals, equipments, glassware and miscellaneous materials required for the study is given in appendix.

4.2 Methodology

4.2.1 Study site

The study was conducted at National Tuberculosis Centre (NTC), Thimi, Bhaktapur, from September 2005 to May 2006.

A total of 295 clinically suspected patients having sputum positive diagnosed by fluorescent microscopy were included in the study. The sputum samples were decontaminated and cultured on 2% modified Ogawa media and smear examination was done from pellet. The *M .tuberculosis* bacteria were confirmed by different biochemical tests. The culture positive isolates were tested for anti-TB drug susceptibility testing by Proportional Method as standard protocol.

4.2.2 Study population

The following groups of individuals were included for this study.

1. New cases of pulmonary tuberculosis visiting NTC who were sputum smear positive in microscopy (Category I)

2. Treatment failure (Category II)

3. Return after default (Category II)

4. Follow-up

5. Chronic cases (Category IV)

4.2.3 Sample collection

4.2.3.1 Sputum

Sputum is the sample of choice in the investigation of PTB in this study. During the sample and date of collection, all the research objectives and the expected outcomes had been briefed and the verbal consent had been taken from each study participant. For the patient suspected to be PTB at the first visit, triplicate sputum samples first, on the spot; second, early morning sample on the next day; and third, on the spot on the same day, were collected at NTC. As for TF, RAD, FU and chronic cases, the sputum was collected on the spot. All the samples were collected in leak proof, wide mouth, transparent, sterile and stopper plastic container. The patients were given clear instruction about the quality and the quantity of the samples. For this, the patients were instructed to rinse his/her mouth with water, standing facing wall, away from wind; keep both hands on his/her hips; cough forcibly; collected sputum in his/her mouth and spitted carefully in the container and closed the lid gently. Adequate safety precautions were taken during the specimen collection to prevent the spread of infectious organism.

4.2.3.2 Sample evaluation

A good sputum sample consists of recently discharged material from the bronchial tree, with minimum amount of oral and nasal material. The sputum specimens were collected such that adequate safety precautions were taken to prevent the spread of the infectious organism. The container was labeled and filled request form available in the NTC. The request form contained patient's full name, age, sex, date and time of collection. About 4 ml mucopurulent sputum was collected (Collee *et al.,* 1999).The specimen was processed within 2 hours or if not processed kept at 4˚C.The collected specimens were

stained by flurochrome method and the sputum positive for *M. tuberculosis* was processed for culture and DST.

4.2.3.3 Sample smear microscopy

A small portion of mucopurulent or bloody material was taken from the container with a wooden stick and transferred to the clean, greese free unscratched slide at one end of which has relevant patient's number. The specimen was spread to the size 2cm×1cm, dried at room temperature for 15min and heat fixed by passing the slide through the flame 3 to 3 times with the smear uppermost and allowed to cool before staining. The entire steps were conducted inside a safety cabinet.

Microscopic examination of direct smear by auramine fluorochrome method

1. The heat fixed slides were placed on the staining rack with smear facing up.

2. The auramine solution was placed on the slide to cover the entire smear and allowed to stand for 20 minutes.

3. After 20 min, the slides were washed with tapwater and drained.

4. The slides were then decolorized with 20% H_2SO_4 for 3-5 min.

5. After 3-5 min, the slides were washed with tapwater and drained.

6. The slides were then treated with methylene blue, counter stained for 30 seconds.

7. The slides were gently washed with tapwater and drained.

8. Then after, the slides were dried in air and the whole smear was examined using 20 × objective in fluorescence microscope.

The microscopic produced bright yellowish objects against a dark background.

Table 3. Interpretation of the Result (Stained smear)

No. of AFB observed/Field	Report
No. AFB found	--
1-2 AFB/300 VF	-+ (report the examination of another smear from the same or another specimen).
3-9 AFB/300 VF	+
10-299 AFB/300 VF	++
More than 300 AFB/300 VF	+++

No.: Number VF=Visual Field AFB=Acid Fast Bacilli

(Source: American Lung Association in USA)

4.3 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. The sputum samples were processed as follows:

4.3.1 Decontamination and digestion of sputum for culture (NaOH Ogawa method)

1. An equal volume of 4% NaOH was added to one volume of sputum specimen contained in a graduated centrifuge tube of 15ml capacity.

2. The cap of the tube was tighten and shaken to digest the sputum.

Then the tube was let to stand for 15 min at room temperature.

4.3.2 Primary culture of Mycobacteria

1. The condensed water in the 2% Modified Ogawa medium contained in the media tube was removed by putting the tube upside down on the spirit cotton.

2. 0.1ml of the digested specimen by NaOH Ogawa method was inoculated onto each of the two culture tubes of Ogawa media slopes.

3. Then the inoculum was spread evenly over the whole surface of each medium.

4. The caps of inoculated medium tubes were loosen and the tubes were laid on the slanting bed so that the slants face were kept upward.

5. The inoculated slants were kept in the incubator at 37˚C.

6. After a few days or more of incubation when the surface of the media has been dried, the caps of incubated medium tubes were tightened and the incubation was continued.

7. Then the culture was observed at one week for rapid growers and at 4 weeks for slow growers. If the colonies did not appear at the 4th week, weekly observation was done until 8 weeks before giving decision as negative.

8. The growth which appeared as colony of dry and irregular margin with buff colour at 4th week of the incubation was recorded and reported.

4.3.3 Reading of primary culture

Table 4. Grading of primary culture based on WHO/IUATLD (2001)

Reading	Report
No growth	Negative
1-19 colonies	Positive(Record the actual number of colonies)
20-100 colonies	Positive(1+)
100-200 colonies	Positive(2+)
200-500 colonies(Almost confluent growth)	Positive(3+)
>500 colonies(Confluent growth)	Positive(4+)
Contaminated	Contaminated

The negative cultures were discarded after 8 weeks. All the suspected colonies were examined by Fluorochrome method for confirmation of the mycobacterial colonies.

4.4 Identification of isolates

Identification of significant isolates were done by using standard microbiological techniques as described in Bergey's Manual of Systemic Bacteriology (1896) which involve morphological appearance of the colonies, staining reaction and biochemical properties.

In primary isolates, growth rate, colony characteristics and fluorochrome staining of organism was observed. The isolates were then subcultured onto 2% Modified Ogawa medium for biochemical tests and onto drug containing medium for drug susceptibility test by Proportion Method.

4.4.1 Biochemical tests

Biochemical tests were conducted from the subculture tube. The colonies on the Ogawa media were further confirmed by conventional biochemical tests (Niacin test, Nitrate test and Catalase test).For biochemical test, a culture must be at least 3 to 4 week old

and must have without any contamination and sufficient growth of 100 or more colonies.

4.4.1.1 Niacin test (By aniline method)

1. 2ml of boiling water was poured onto the media. The tube was kept at slanting position for 10 minutes to cover whole surface of medium with boiling water.

2. Two tubes were prepared and 0.2ml of the extract was put into each of the tubes.

3. 0.1ml of 4% aniline ethanol was added in each tube.

4. 0.1ml of 10% cynogen bromide solution was added into one of the tubes and mixed them gently. The rest tube was kept as control. The high percentage was to be undertaken to avoid any inhalation of the vapour of carcinogenic cynogen bromide. This procedure was done in well ventilated cabinet.

As the result, yellow colour indicated the presence of Niacin; and hence the presence of *M. tuberculosis*.

Detoxification of cynogen bromide

Cynogen bromide is poison.Cynogen bromide in the tube should be detoxified by adding 0.2ml of alkali(4% NaOH) into the tube after the test, then autoclave and wash the tube (Bacteriology Examination to Stop TB,Aoka Fujiki).

4.4.1.2 Nitrate test

Procedure

a. A heavy growth culture tube was taken.

b. About 2.5ml of sodium nitrate phosphate buffer (solution No. 1) was added and the tubes were incubated for 2 hours at 37˚C at slant position. The colonies were covered by the buffer.

c. After 2 hours, the extract on the tube was acidified with one drop of H_2SO_4(10%) added two drops of 0.2% Sulfanilamide(Solution No.3), and two drops of 0.1% N-Napthylethylenediamine.

d. The tubes were observed, formation of pink colour indicating nitrate positive test.

H37RV strain was used as positive control.

4.4.1.3 Catalase test

Heat labile test (68^0c, PH7.0) for identification of M. tuberculosis

1. With a sterile pipette, aseptically 0.5ml of 0.06 M phosphate buffer, PH 7.0 to 16 x 125 mm screw cap tubes.
2. Several loopfulls of test cultures were suspended in the buffer solution using sterile loops.
3. The tubes containing the emulsified cultures were placed in a previously heated water bath at 68^0 C for 20 minutes. Time and temperature was critical.
4. The tubes were removed from the heat and allowed to cool to room temperature.
5. 0.5ml of freshly prepared Tween-peroxide was added to each tube and caps were replaced loosely.
6. Then it was observed for the formation of bubbles appearing on the surface of the liquid. The tubes were not shaken as Tween 80 may form bubbles when shaken, resulting in false positive result.

   The negative tubes were held for 20 minutes before discarding.

4.5 Drug susceptibility testing

Drug susceptibility test was done on *M. tuberculosis* isolate from each patient by Proportion method as standard protocol. Each strain was tested against four antibiotics at the following concentrations: Isoniazid (INH) at 0.25µm/ml and 8µm/ml; Ethambutol (EMB) at 1µm/ml and 2µm/ml; Streptomycin(SM) at 4µm/ml and 8µm/ml and Rifampicin (RMP) at 20µm/ml and 40µm/ml.

4.5.1 Proportion method for DST (simplified variant)

The LJ medium is used for all the resistance tests. Working drug dilutions should be prepared on the day of use; 1 ml of working solution added to 500 ml of LJ medium will yield final drug concentrations equivalent to the different critical concentrations. The medium is distributed in volumes of 6–8 ml in sterile 17 mm x 170 mm screw-capped testtubes, coagulated at 85 °C for 45 minutes, and allowed to cool at room temperature for 24 hours; the screw caps are then tightened and the tubes stored at 4 °C. The control medium without drugs is prepared at the same time as the drug-containing media. The period of validity of the media stored at 4 °C is 2 months.

4.5.2 Preparation of inoculum/bacillary suspension

1. A loopful of colonies was harvested from the Ogawa media and suspended on the one or two drops of distilled water in a test tube. The tube contained several (5-6)5-6mm sized sterile glass beads.
2. The tube was vortexed on vortex mixer for homogenization for few min and left for 10 min.
3. 7ml of sterile distilled water was added; the supernatant suspension was transferred to another sterile tube and its density/turbidity was adjusted with

sterile water to that of 1% MacFarland No. 1 suspension. This constitutes 'the neat suspension'.

4. Serial dilution was made with the neat suspension by transferring 0.1ml of the neat suspension to the tube containing 0.9ml distilled water and mixed well (suspension-1 with 10^{-1} dilution).
5. About 0.1ml of suspension-1 was transferred to the tube containing 0.9ml distilled water mixed well (suspension-2 with 10^{-2} dilution).
6. Finally, 0.1ml of the suspension-2 was transferred into the tube containing 0.9ml distilled water and mixed well (suspension-3 with 10^{-3} dilution).

4.5.3 Preparation of drug media

The preparation of the drug containing media for the Proportion method was described in the Table 5.

Table 5. The preparation of drug containing media for the Proportion method

Concentration in culture media(µg/ml)	0.25	1	2.0	4.0	8.0	20.0	40.0
INH	√				√		
SM				√	√		
RMP						√	√
EMB		√	√				

4.5.4 Inoculation of bacillary suspension/inoculum

1. About 8 media tubes containing different concentrations of anti-TB drugs shown in the Table No. 5 were taken. Each anti-TB drugs having different concentration was incorporated into 2 media tubes.

2. Suspension-1 was inoculated to all these 8 drug-media slopes by using sterile inoculating loop to each media. Similarly, one loopful suspension-1 was inoculated into Control 1(Plain LJ media); one loopful suspension suspension-2 into Control 2 and one loopful suspension 3 into Control 3.

3. The suspension was spread over the whole surface of the medium and the caps loosely closed. The tubes were placed at slanting position in the incubator for 24 hours and then in upright position with tighten cap. Then all the tubes were incubated at 37˚C for 4 weeks and finally the results were interpreted.

4.5.5 Interpretation of results

Control I: It shows the unhindered growth of that bacterial suspension, which was inoculated on the culture media with strong (mostly bactericidal) drugs: INH, SM, RMP and EMB.

Control III: The growth on this culture media is equivalent to the "critical" proportions of both bacteria suspensions I + II (1% of Control I, 10% of Control II). The growth density (if possible no. of colonies) was compared on the culture media with critical concentration of the corresponding drug with the growth on Control III.

Table 6. Critical concentrations of drugs

S.N.	Drugs	Concentrations
1	INH	0.25µg/ml
2	SM	4.0µg/ml
3	RMP	40.0µg/ml
4	EMB	2.0µg/ml

Source: NTC,2005

Sensitive(S)

A strain of TB bacteria was termed sensitive, when there was no growth on the culture media with the "critical" concentration of the respective drug.

Resistant(R)

A strain of TB bacteria was termed resistant, when the growth on the culture media with the "critical" concentration of the respective drug was equal or stronger than on Control III.

Questionable (Q)

The test 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 start.

4.6 Quality control for the tests

Quality assurance

To ensure that results of susceptibility testing are reliable and comparable between different countries, a system of quality assurance is recommended. Susceptibility testing should be quality-controlled on three levels – internal, national, and international.

Internal quality control of susceptibility testing

Susceptibility testing should be performed on the standard *H37Rv* strain in each new batch of LJ medium and for each drug. It is also recommended that this internal quality control also include a known drug-resistant strain. Standardized procedures should be followed whether the proportion method, BACTEC®, resistance ratio, or other method is used for susceptibility testing and for formulation of media. As a part of internal quality control, the quality of the medium should be controlled for each batch. Drugs added to the medium must be pure drugs obtained from a reputable firm and the

percentage of potency must be clearly indicated. Drug dilutions and their addition to the medium should be performed following accepted standards.

To maintain quality control in this study, strict aseptic condition was maintained during collection and processing of the sample. The sterility of each batch of the test medium was confirmed by incubating one uninoculated tube with the inoculated tubes as quality control.

CHAPTER 5

5. RESULTS

This study was carried out by Central Department of Microbiology in collaboration with National Tuberculosis Centre (NTC), Thimi, and Bhaktapur during September 2005 to May 2006.A total of 295 suspected PTB cases attending at NTC was included in the study. Among 295 suspected cases, 250 were previously treated (old cases) and 45 were untreated cases (new cases). The sputum samples from patients under study were subjected to test for auramine fluorochrome staining, culture and antibiotic susceptibility testing for culture positive isolates.

5.1. Age and sexwise distribution of the PTB patients attending NTC

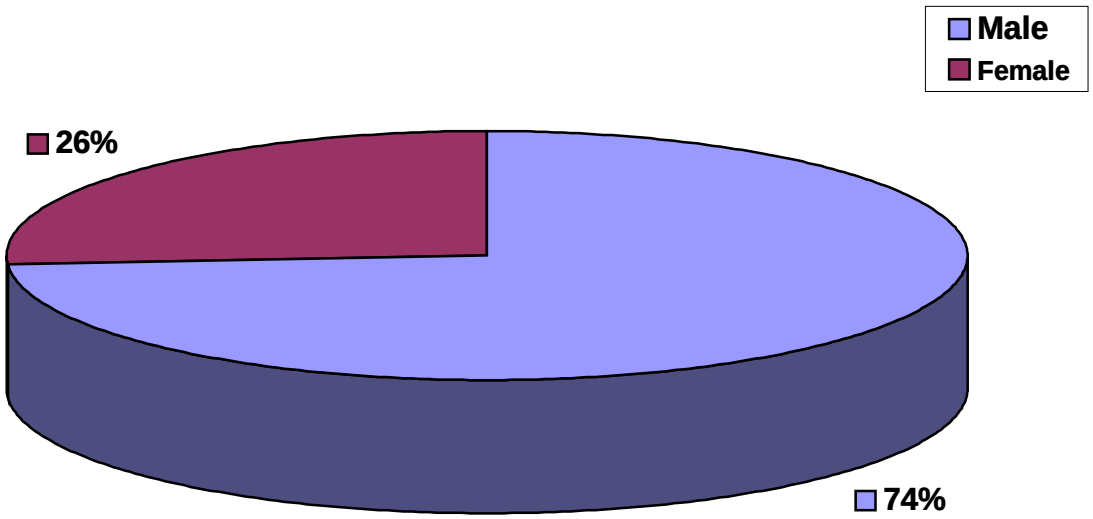


Fig 1. Sexwise distribution of pulmonary TB patients enrolling in the study

Table 7. Age and sexwlse distribution of PTB patients studied

S.N.	Age group	Male		Female		Total	
		No.	%	No.	%	No.	%
1	11-20	19	6.44	18	6.10	37	12.54
2	21-30	62	21.02	23	7.79	85	28.81
3	31-40	45	15.25	16	5.42	61	20.68
4	41-50	39	13.22	13	4.41	52	17.63
5	51-60	21	7.12	5	1.69	26	8.81
6	61-70	24	8.14	3	1.02	27	9.15
7	71-80	6	2.03	0	0.00	6	2.03
8	Above 80	1	0.34	0	0.00	1	0.34

Among the studied 295 cases, 73.56 % (n=217) were male and 26.44 % (n=78) were female in the age group from 11 year to 88 year. The study showed that the highest number was seen in the age group 21-30, followed by 31-40.

5.2 Antibiotic susceptibility pattern of *M. tuberculosis* isolates

Among 295 *M. tuberculosis* isolates,41.69% (n=123) were sensitive to INH;59.66% (n=176) were sensitive to SM;60.33% (n=178) were sensitive to RMP; and 66.10% (n=195) were sensitive to EMB.

Table 8 Pattern of antibiotic sensitivity of isolates

S.N.	Antibiotics	No. of isolates	Sensitive		Resistant	
			No.	%	No.	%
1	INH	295	123	41.69	172	58.30
2	SM	295	176	59.66	119	40.33

3	RMP	295	178	60.33	117	39.66
4	EMB	295	195	66.10	100	33.89

Similarly,58.30% (n=172)were resistant to INH; 40.33% (n=119) were resistant to SM;39.66% (n=117) were resistant to RMP; and 33.89% (n=100) were resistant to EMB.

5.3 Antibiotic susceptibility pattern in patients with or without past history of treatment

Among 295 cultures positive *M. tuberculosis* isolates studied from drug susceptibility testing, resistance to one or more anti-drugs were exhibited.

Out of 295 isolates, 45 isolates were from untreated patients and 250 isolates were from treated patients.

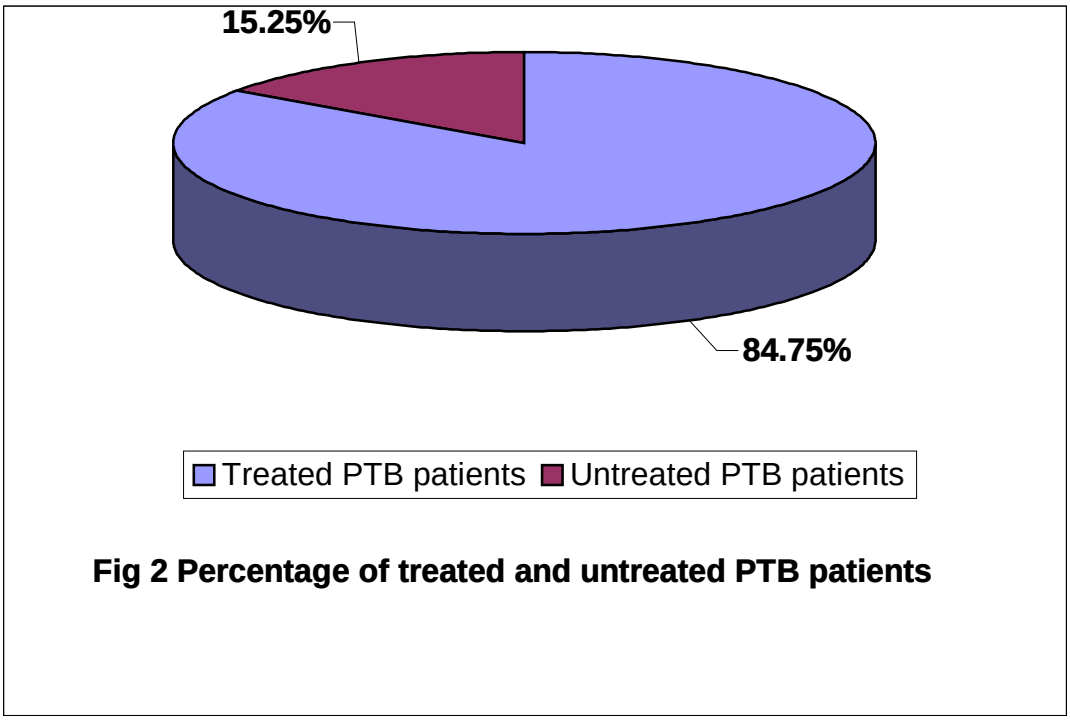


Fig 2 Percentage of treated and untreated PTB patients

Among the untreated patients, 35.55 % (n=16) were sensitive to all the 4 drugs whereas the remaining were resistant to two or more drugs. Resistant to only one drug was shown by 20 % (n=9) of the isolates .Resistant to two drugs was shown by 17.77 % (n=8), three drugs to 11.11 % (n=5) and four drugs to 6.66 % (n=3) of the isolates.

Table 9 Antibiotic susceptibility pattern in patients with or without past history of treatment

S.N.	Drug susceptibility testing result	Untreated TB patients		Treated TB patients		Total	Total in Percent
		No.	%	No.	%		
1	Total tested	45	15.25	250	84.74	295	
2	Sensitive to all 4 Drugs	16	35.55	63	25.20	79	26.77%
3	Resistant to 1 Drug						
	INH	6	13.33	35	14.00	41	13.89%
	SM	3	6.66	16	6.40	19	6.44%
	RMP	0	0	1	0.40	1	0.33%
	EMB	0	0	7	2.80	7	2.37%
4	Resistant to 2 Drugs						
	INH+RMP	2	4.44	9	3.60	11	3.72%
	INH+SM	1	2.22	5	2.00	6	2.03%
	RMP+SM	3	6.66	3	1.20	6	2.03%
	INH+EMB	2	4.44	14	5.60	16	5.42%
	SM+EMB	0	0	0	0	0	0%
5	Resistant to 3 Drugs						
	INH+RMP+SM	3	6.66	24	9.60	27	9.15%
	RMP+SM+EMB	0	0	0	0	0	0%
	RMP+INH+EMB	2	4.44	11	4.40	13	4.40%
	INH+SM+EMB	0	0	6	2.40	6	2.03%

6	Resistant to all Drugs	3	6.66	47	18.80	50	16.94%
7	Multi-Drug Resistance (MDR) at least INH + RMP	10	22.22	93	37.20	103	34.91%

Out of the 250 treated TB patients, 25.20 %(n=63) were sensitive to all the four anti-TB drugs and the remaining187 isolates were resistant to one or more drugs. Resistant to one drug was found in 23.6 % (n=59); two drugs to 12.4 % (n=31) and three drugs to 16.4 % (n=41).

Multi drug resistance (MDR) in untreated TB patients were found in 22.22 % (n=10) and in treated TB patients was 37.20 % (n=93).

Table 10. Antibiotic susceptibility pattern in patients with past history of treatment

S.N.	Drug susceptibility testing result	Relapse		Chronic		Follow-Up		Default		Treatment failure	
		No.	%	No.	%	No.	%	No.	%	No.	%
1	Total tested	171	68.4	45	18	19	7.6	8	3.2	7	2.8
2	Sensitive to all 4 Drugs	45	26.31	5	11.11	6	31.57	4	5	2	28.57
3	Resistant to 1 Drug										
	INH	28	16.37	3	6.66	2	10.52	1	12.5	1	14.28
	SM	13	7.60	2	4.44	0	0	1	12.5	0	0
	RMP	1	0.58	0	0	0	0	0	0	0	0
	EMB	9	5.26	1	2.22	0	0	0	0	0	0
4	Resistant to 2 Drugs										
	INH+RMP	5	2.92	0	0	1	5.26	0	0	1	14.28
	INH+SM	3	1.75	0	0	0	0	1	12.5	0	0
	RMP+SM	3	1.75	1	2.22	0	0	0	0	0	0
	INH+EMB	12	7.01	1	2.22	0	0	0	0	0	0
	SM+EMB	0	0	0	0	0	0	0	0	0	0

5	Resistant to 3 Drugs										
	INH+RMP+SM	12	7.01	10	22.22	1	5.26	0	0	1	14.28
	RMP+SM+EMB	1	0.58	0	0	0	0	0	0	0	0
	RMP+INH+EMB	5	2.92	6	13.33	0	0	0	0	0	0
	INH+SM+EMB	2	1.16	2	4.44	1	5.26	0	0	0	0
6	Resistant to all Drugs	25	14.61	13	28.88	7	36.84	1	12.5	1	14.28
7	Multi-Drug Resistance (MDR) at least INH+RMP	47	27.48	29	64.44	9	47.36	1	12.5	3	42.85

Among 250 treated cases,68.4% (n=171) were relapses;18% (n=45) were chronic;7.6%(n=19) were follow-up;3.2% (n=8) were defaulters; and 2.8% (n=7) were Treatment failure PTB cases.

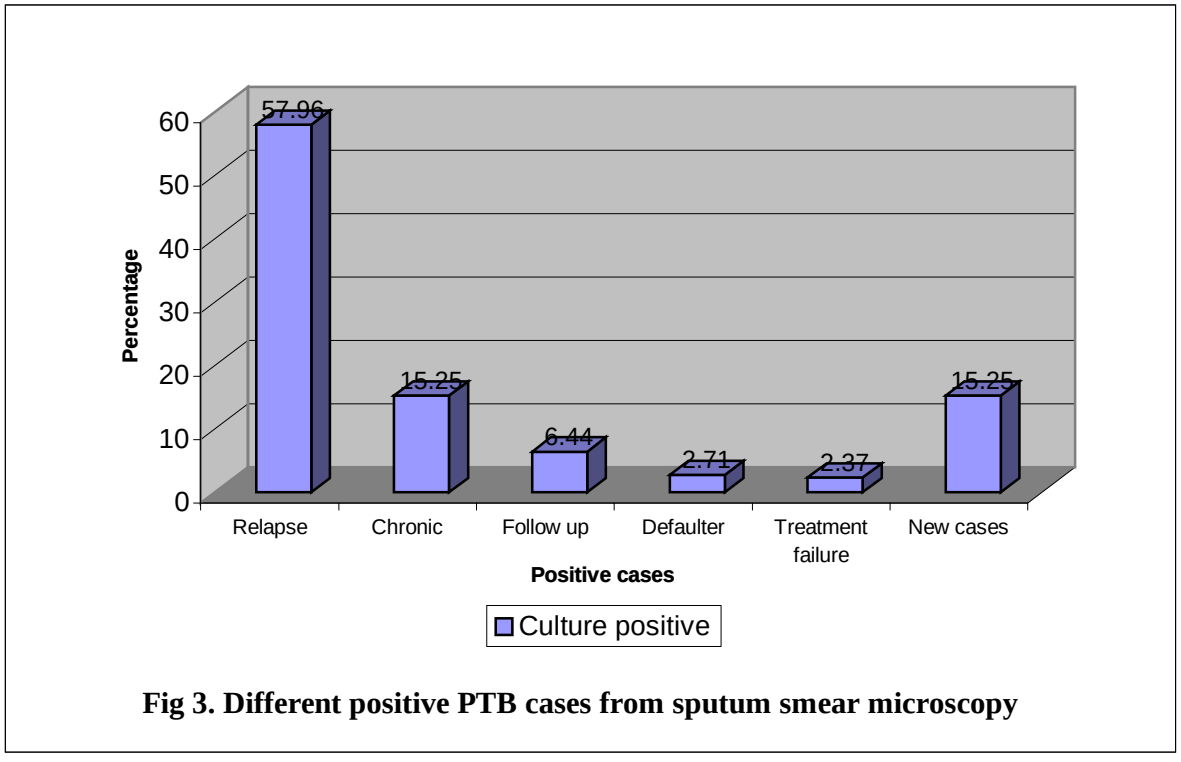


Fig 3. Different positive PTB cases from sputum smear microscopy

The highest percentage of MDR TB was from chronic (64.44%) followed by follow-up (47.36%), treatment failure (42.85%), relapse (27.48%) and defaulter (12.5%).

5.4 Primary and acquired anti-TB drug resistance

Table 11. Comparison of primary and acquired anti-TB drug resistance.

Drug resistance	1 Drug	2 Drugs	3 Drugs	4 Drugs	MDR
Primary drug resistance (n=45)	20.00	17.77	11.11	6.66	22.22
Acquired drug resistance (n=250)	23.60	12.40	16.40	18.80	37.20

The primary drug resistance (PDR) to one drug was 20%,to two drugs was 17.77%,to 3 drugs was 11.11%, to four drugs was 6.66% and primary MDR was in 22.22% of the isolates.

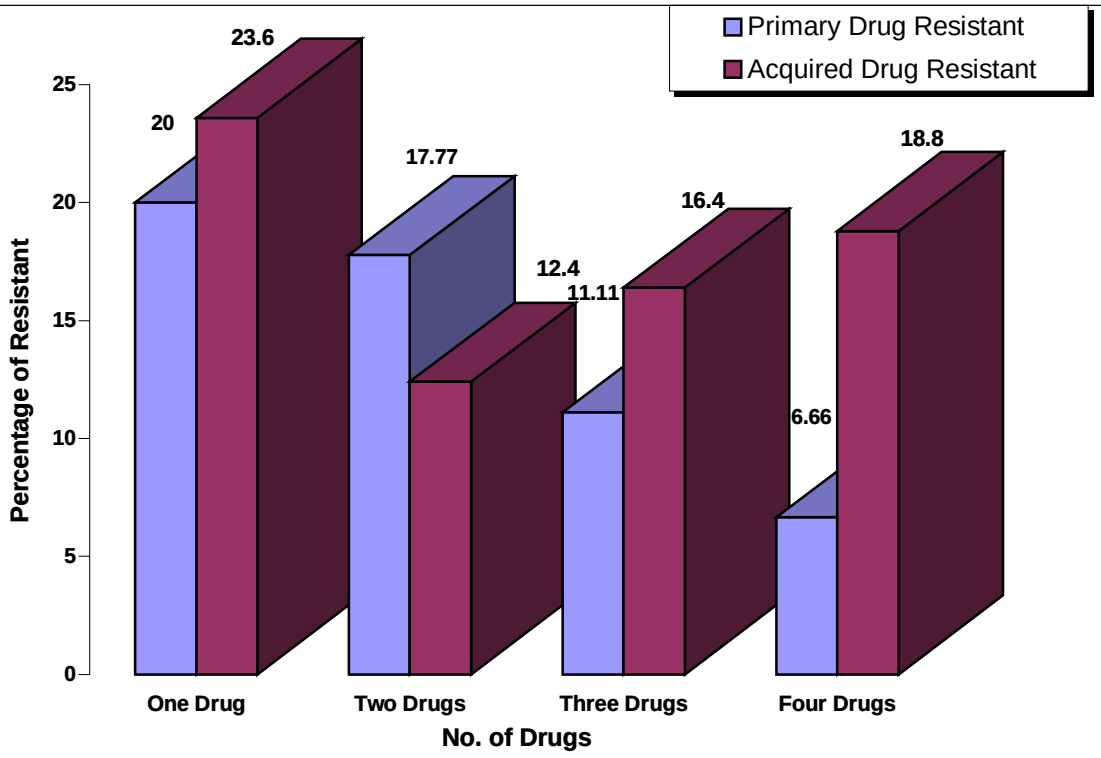


Fig 4. Pattern of drug resistance in primary and acquired drug resistance

Similarly, acquired drug resistance to one drug was 23.60%,to two drugs was 12.40%, to three drugs was 16.40% , to four drugs was 18.80% and acquired MDR was in 37.20% of the isolates.

5.5 Age and sexwise distribution of resistant *Mycobacterium* spp.

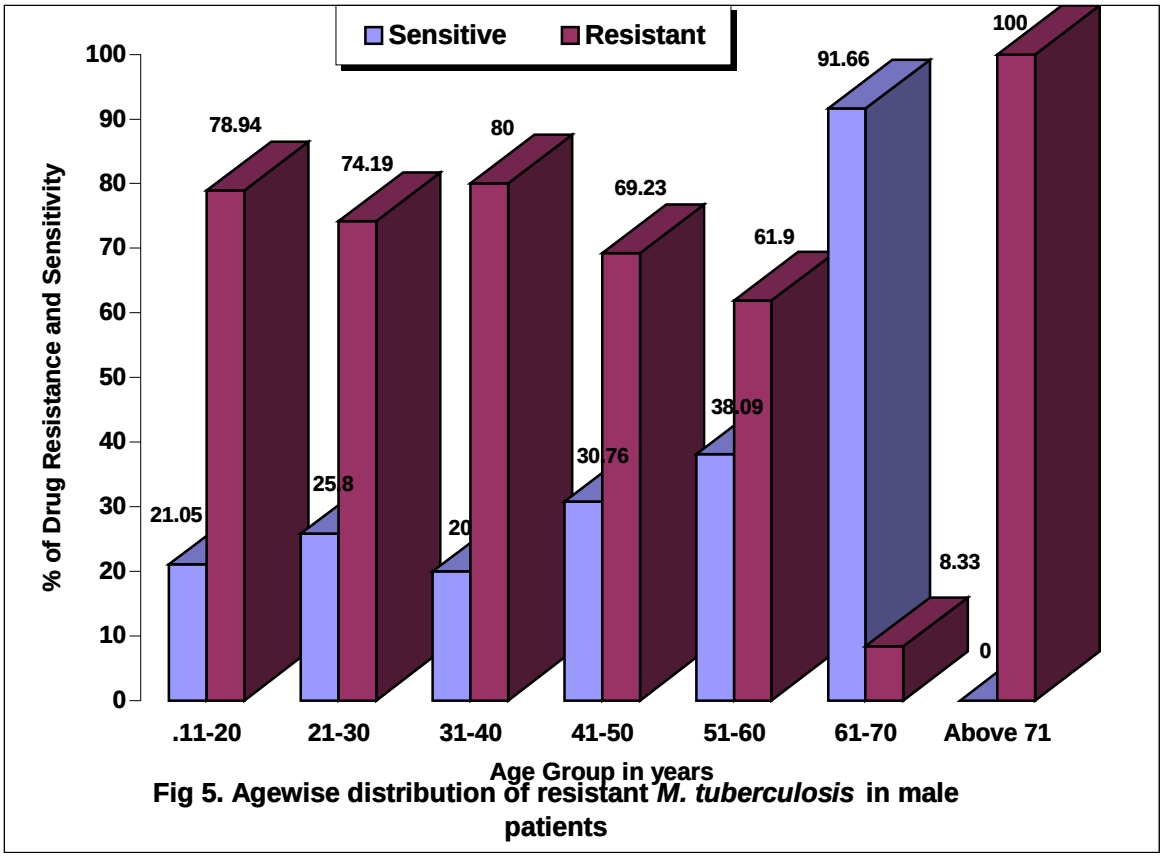


Fig 5. Agewise distribution of resistant *M. tuberculosis* in male patients

Table 12. Agewise distribution of resistant *M. tuberculosis* in male

S.N.	Age group	No. of isolates	Sensitive		Resistant	
			No.	%	No.	%
1	11-20	19	4	21.05	15	78.94
2	21-30	62	16	25.80	46	74.19
3	31-40	45	9	20.00	36	80.00
4	41-50	39	12	30.76	27	69.23
5	51-60	21	8	38.09	13	61.90
6	61-70	24	22	91.66	2	8.33
7	Above 71	7	0	0.00	7	100
Total		217	71		146	

The highest number of *Mycobacterium spp* was found to be maximum in age group above 71 i.e. 100% (n=7) followed by 80% (n=36) in 31-40 age group,78.94% (n=15) in age group11-20,74.19% (n=46) in 21-30 age group,69.23% (n=27) in 41-50 age group,61.90% (n=13) in 51-61 age group and 8.33% (n=2) in age group 61-70 in case of male.

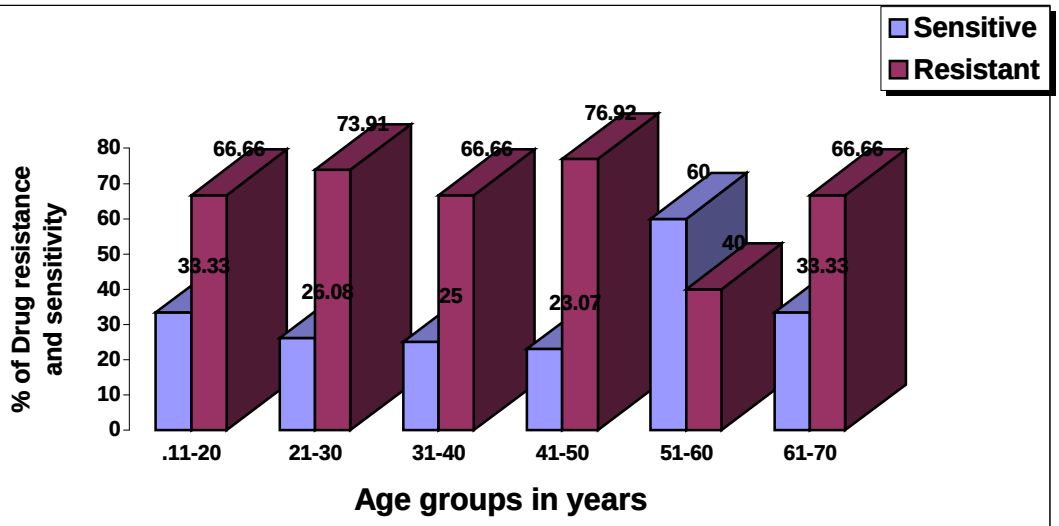


Fig 6. Agewise distribution of resistant *M. tuberculosis* in female patients

Table 13. Agewise distribution of resistant *M. tuberculosis* in female

S.N.	Age group	No. of isolates	Sensitive		Resistant	
			No.	%	No.	%
1	11-20	18	6	33.33	12	66.66
2	21-30	23	6	26.08	17	73.91
3	31-40	16	4	25.00	12	66.66
4	41-50	13	3	23.07	10	76.92
5	51-60	5	3	60.00	2	40.00
6	61-70	3	1	33.33	2	66.66
Total		78	23		55	

In case of female patients, the highest number of resistant was found in age group 41-50 76.92% (n=10) followed by 73.91% (n=17) in 21-30 age group, 66.66% (n=12) in age group11-20 , 31-40 and 61-70,40% (n=2) in 51-60 age group.

The study demonstrated that age and sex were not significantly related to drug resistance (P<0.05).

5.6 Distribution of drug susceptibility of *M. tuberculosis* in patients with family history of TB

Table 14. Distribution of drug susceptibility of *M. tuberculosis* in patients with family history of TB

S.N.	Drug susceptibility	No. of isolates	Family history of TB patients				Total	
			Present No.	%	Absent No.	%	No.	%
1	Drug resistance	71	21	29.57	50	70.42	71	47.33
2	Drug sensitive	79	32	40.50	47	59.50	79	52.66
	Total	150	53		97		150	

Among 295 PTB cases, 150 cases were randomly selected and interviewed. Among 79 drug sensitive isolates isolated from PTB patients, 40.50 % (n=32) were isolated from those patients who had previous cases of TB in his/her family and 59.50 % (n=47) had no previous cases of PTB in his/her family.

Among 71 drug resistant isolates isolated from PTB patients 29.57 % (n=21) had previous cases of PTB in his/her family and 70.42 % (n=50) had no previous PTB cases in his/her family.

The study revealed that there was not significant relation between the drug resistance and family history of TB patients (P<0.05).

5.7 Distribution of drug susceptibility of *M. tuberculosis* in patients with habit of taking alcohol

Table 15.Distribution of drug susceptibility of *M. tuberculosis* in patients with habit of taking alcohol

S.N.	Drug susceptibility	No. of isolates	Alcoholic		Non-alcoholic		Total	
			No.	%	No.	%	No.	%
1	Drug resistance	71	25	35.21	46	64.78	71	47.33
2	Drug sensitive	79	23	29.11	56	70.88	79	52.66
	Total	150	48		102		150	

Among 79 drug sensitive isolates isolated from PTB patients, 29.11 % (n=23) were isolated from those patients who were found to be alcoholic and 70.88 % (n=56) were found to be non-alcoholic.

Among 71 drug resistant isolates isolated from PTB patients 35.21 % (n=25) were found to be alcoholic and 64.78 % (n=46) were found to be non-alcoholic.

The study revealed that the habit of taking alcohol and drug susceptibility pattern were independent to each other ($P<0.05$).

5.8 Distribution of drug susceptibility of *M. tuberculosis* in patients with habit of smoking.

Table 16. Distribution of drug susceptibility of *M. tuberculosis* in patients with habit of smoking

S.N.	Drug Susceptibility	No. of isolates	Smokers		Non-smokers		Total	
			No.	%	No.	%	No.	%
1	Drug resistance	71	28	39.43	43	60.56	71	47.33
2	Drug sensitive	79	37	46.83	42	53.16	79	52.66
	Total	150	65		85		150	

Among 79 drug sensitive isolates isolated from PTB patients, 46.83 % (n=37) were isolated from those patients who were found in smoking habit and 53.16 % (n=42) were found to be in non-smoking habit.

Among 71 drug resistant isolates isolated from PTB patients 39.43 % (n=28) were found to be in smoking habit and 60.56 % (n=43) were found to in non-smoking habit.

The study demonstrated that the smoking habit and drug susceptibility pattern was independent to each other ($P<0.05$).

CHAPTER 6

6. DISCUSSION

Tuberculosis has become a grave concern in all part of the world because of recent resurgence of TB.Reasons of this resurgence have been identified by WHO as mainly due to HIV pandemic, less health priority given to the disease and significant increase of multidrug resistant tubercle bacilli as a result of inadequate treatment. The causative agent was discovered more than 100 years ago and highly effective drugs and vaccines are available making TB a preventable and curable disease. It remains as the most significant cause of morbidity and mortality due to a single infectious agent in the world.

Tuberculosis is one of the major public health problems in the third world countries with approximately estimated 60% of the adult population being infected with TB. In Nepal, about 45% of the total population is infected with TB. Every year 40,000 people develop active TB of whom 20,000 have infectious pulmonary disease. Nepal, by estimated number of cases, is ranked at 27 globally (Source: Global TB Report 2005).Despite the expansion and implementation of a much improved National Tuberculosis Programme(NTP) through DOTS Strategy throughout the country,6000-7000 people still die from TB each year in Nepal.

The main objective of this study is to know the resistance pattern of the anti-TB drugs in PTB patients. A total 295 cases were included in this study carried out from September 2005 to May 2006. Out of 295 cases, 250 cases were previously treated cases and 45 were untreated cases. During this study, among the 295 cases of TB, 73.89 %(n=218) males were found higher in number than female 26.10%(n=77) in age group from 11 to above 80 year. The highest number of cases belonged to the age group 21-30 (29.81%).This finding was concordant with similar studies in other countries. In a similar study in Italy, Ponticiella *et al*.(1997) reported ,82.2% males and 17.8% of

females among 90 active PTB cases; Blumberg *et al*(1991-1997) in Atlanta, USA reported 74% of the male and 26%of the female TB cases among 1536 cases. Likewise, in Archangeh, Russia, Toungoussova *et al.*(2002); in Korea reported 66.49% if the male and 34.31% of the female TB cases among 2486 cases. Kuban *et al.,* (2002) in Cameroon, Yaunde, reported 65.76% of male and 34.25% of the female TB cases among 111 cases; Riantawan *et al.,* (1997) in Thailand reported 77% male and 23% of the female cases among 1441 cases. Tuberculosis Control Programme, Nepal reported 66.77% male and 33.23% female of TB cases among 14,384 newly diagnosed TB cases during 2002/2003.All above findings are consistent with this study.Shrestha *et al*.,1996 ,reported 47% males and 3.05% of female TB cases in histopathological specimens at Tribhuvan University Teaching Hospital. Smith (1996) reported that as in most countries of the world, in Nepal, the reported incidence of TB is higher in man than women.Rijal (2004) reported that the study conducted at NTC, among the 325 cases, 75.69% were male and 24.30% were female. Thus the incidence of TB was found higher in male than female patients; male patients are affected more than female patients. According to the significance test, the prevalence of TB in male and female was found to be statistically significant. These possible factors explain the gender differences observed, the most commonly accepted being that women are less exposed to infection than men. The second might be the biological difference, such as an increased susceptibility in male.Finally,infected women may progress more frequently to disease and die more rapidly, leaving a cohort with a low prevalence of infection.

In this study, out of 295 culture positive isolates, 15.25 %(n=45) isolates from the untreated TB cases and 84.74% (n=250) isolates from the treated TB cases. The study showed that 26.77 %(n=79) were sensitive to all the four drugs and 68.81 %(n=203) cases were drug resistant to one or more drugs. Monoresistance to INH, SM, RMP and EMB was detected in 41(13.89%), 19(6.44%), 1(0.33%) and 7(2.37%) strains respectively; and 23.05% to a total single drug resistance. Similarly, resistance to two drugs was detected in 39 strains(13.22%);resistant to three drugs was detected in 46

strains (16.94%).The level of Multi-drug resistant cases were found to be 34.91%(22.22% Primary MDR and 37.20% Acquired MDR).

The highest rates of drug resistance were discovered for isoniazid and streptomycin. In both new and treated cases, 13.33% and 14% of the strains isolated were resistant to isoniazid respectively; whereas 6.66% and 6.40% of the strains isolated from new and previously treated patients were resistant to streptomycin respectively.

The initial drug resistance case was found in 8.47% to one or more of the four anti-TB drugs was found to be 60.33%.In addition, the primary drug resistance(PDR) to one drug was 20%,to two drugs was 17.77%,to 3 drugs was 11.11% and to four drugs was 6.66%.And the acquired drug resistant to one drug was 23.60%, to two drugs was 12.40%, to three drugs was 16.40% and to four drugs was 18.80%.The primary MDR was found to 22.22% of the isolates and the acquired MDR was in 37.20% of isolates. This finding of this study is similar with the latest third surveillance report of WHO 2004 in Nepal. The report revealed that 1.3% and 20.5% of the new and old cases had MDR in Nepal. The rate of acquired MDR-TB was higher (19.25%) than the rate of primary MDR-TB (2.63%).

Out of 295 culture positives isolates obtained from the PTB patients,171 isolates were from relapse cases,45 isolates from chronic cases,19 isolates from follow-up cases,8 isolates from default cases and 7 isolates from treatment failure cases. The highest percentage of MDR was obtained from the chronic cases (64.44%) followed by follow-up cases (47.36%), treatment failure cases (42.85%), relapse cases (27.48%) and default cases (12.5%).

The findings of this study is in agreement with other studies conducted at different places.Al Marri (2001) in the state of Qatar reported that 85% of the cases were sensitive to anti-TB drugs and 15% cases were resistant to one or more anti-TB drugs among 406 cases of PTB.In West Province of Cameroon, the level of initial drug resistance was found in 14.28% and acquired drug resistance was found in 0.74%,the

rate of MDR was found 0.98%(0.49%initial MDR and 1% for acquired MDR);Kuban *et al.*(2000) reported 4.1%MDR case among 566 isolates,15.2% initial drug resistance and 11.6% acquired drug resisitance and 1.06% and acquired MDR cases 3%).Indian studies showed that 3.4% of the new cases and 25% of the old cases had MDR-TB(WHO,2000).The anti-TB drug sensitivity test conducted in Nepal 1987-1990 revealed that 1.6% and 9.6% of the new and old cases respectively had MDR-TB.GC *et al.*(2001) found 8.57% of initial MDR cases and 100% of acquired MDR cases;Bhattarai *et al.* (2003) obtained 4.16% of primary MDR cases and 5% of acquired MDR cases.Similarly,Rijal *et al.* (2003) found primary MDR in 2.63% of the isolates and acquired MDR in 19.25% of isolates. The alarming increment in MDR-TB cases may be owing to late identification of suspected MDR –TB cases. Identification of all cases of MDR-TB would require culture and susceptibility testing of tuberculosis suspects, an ideal that is unachievable in developing countries like Nepal. The culture and sensitivity facilities for *M.tuberculosis* in our country are country are only in National Tuberculosis Centre (NTC) and Germany-Nepal Tuberculosis. Project (GENETUP).Other reasons for increase in MDR-TB may have smear negative TB and hence may even remain undiagnosed with tuberculosis. Even among those with smear positive disease, initial response to treatment may be good, and MDR-TB may not be suspected in some cases.

This study revealed that there were high rates of initial drug resistance against isoniazid and streptomycin among both new and treated cases .This may be due to low cost and widespread use in the treatment of TB. The most significant finding of our study was the low frequency of primary resistance to rifampicin which is a good indicator for success of DOTS. Monoresistance to Rifampicin was not observed at all in new cases. Resistance to Rifampicin predicted resistance to isoniazid and streptomycin and served as marker of MDR.

The high rates of resistance among new cases indicated that drug resistant strains are circulating and are being transmitted from patient to patient in our country Nepal.

Transmission of already resistant strains as a serious problem and threat, as it is different to treat patients infected with drug resistance, it is important for a TB control programme to have reliable laboratory facilities for susceptibility testing of *M.tuberculosis* isolates.

This study showed there were no any relation of age and sex, with drug resistance.The result of the present study was in agreement with the study of Al-Marri (2001) in Qatar; Warndroff *et al.*(2000) in Karongo District,Malavi also supports our study that neither nor acquired drug resistance were associated with sex or age.

Likewise, the family history of TB cases, smoking and alcoholic habit of the patients did not show any significant relation with drug resistance. This study was similar to the study of Leung (2001) in Hong Kong; and Toungoussova *et al.*, 2002; Archangels in Russia.

Increase cases of MDR-TB are a global problem. MDR-TB can be cured by the effective implementation of DOTS strategy. Regular monitoring of MDR-TB and policy in accordance with the operational research finding enables the controls and drug quality assessment is helpful for emerging MDR-TB.

In 1997 the World Health Organization (WHO), the International Union Against Tuberculosis and Lung Disease (IUATLD) and partners world-wide released the first report of the global project on anti-tuberculosis drug resistance. The data generated in this report were reinforced in a recently published second report.Directly observed treatment short-course (DOTS), the WHO strategy for TB control cures virtually all patients with drug-susceptible TB and some drug resistant TB through the administration of short-course chemotherapy with first-line drugs.

However, patients with multidrug-resistant (MDR) tuberculosis (TB) to at least isoniazid and rifampicin are more likely to fail short-course chemotherapy. In recent years there has been encouraging evidence that patients with MDR TB can be cured

with appropriate management based on second-line drugs. Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs and reliable assessment of drug resistance is an essential prerequisite for appropriate use. Treatment is prolonged and significantly more expensive. Accurate laboratory drug susceptibility testing (DST) data to second-line drugs will support clinical decision making and help to prevent the emergence of further drug resistance in patients with MDR TB. In order to meet the challenges posed by MDR TB, the WHO established the DOTS-Plus initiative to assess the feasibility and cost-effectiveness of using second-line drugs to manage patients with MDR TB primarily in middle and low-income countries.

DOTS-Plus is needed in areas where MDR-TB has emerged due to previous inadequate TB control programmes. Therefore, DOTS-Plus pilot projects are only recommended in settings where the standard DOTS strategy is fully in place to protect against the creation of further drug resistance.DOTS-Plus is designed to cure MDR-TB using second-line TB drugs. These drugs should be stored and dispensed at specialized health centers with appropriate facilities and well-trained staff. It is *vital* that DOTS-Plus pilot projects follow WHO recommendations in order to minimize the risk of creating drug resistance to second line TB drugs. DOTS-Plus works as a supplement to standard DOTS-based TB programmes already in place.

If patients failing DOTS are presumed to have MDR-TB, and if drug-susceptibility testing is limited, they might be placed on an empirical treatment regimen consisting of second-line TB drugs. Under DOTS Plus, they must endure an additional two years of daily, observed combination therapy, including injectable antibiotics, which can produce unpleasant side-effects. As of July 2002, the Green Light Committee (GLC) had approved seven pilot projects to implement the DOTS-Plus strategy, and is currently reviewing five further applications. Preliminary results from those programmes already under way show percentages of culture negativization to be between 46 and 79 percent. Continued support for these projects – together with the

implementation of new programmes in other countries – will contribute to the building of a sound policy for the control of MDR-TB.

Estonia's country-wide DOTS-Plus programme began in March 2001, and allows for the enrolment of 200 patients over a two-year period. Preliminary results show a sputum negativization of 46 percent of patients after six months of treatment. The pilot project has become a leverage tool to promote the expansion of the DOTS strategy in Estonia.

Latvia began to implement DOTS fully in 1997. However, poor case management in the past and the overcrowded conditions of TB wards still helped to make Latvia the country with the second highest MDR-TB rate in the world. The proportion of MDR-TB among new TB patients in this country is 9.5 percent. On February 2001, the GLC approved a countrywide DOTS-Plus pilot project.

Strengthening MDR-TB control *now* through DOTS-Plus will help to reduce morbidity, mortality and transmission due to MDR-TB. By directing MDR-TB patients to effective treatment protocols *now*, we are saving direct costs. And by controlling the primary cycle of MDR-TB transmission *now*, we are saving future funds and indirect costs that would otherwise have to be diverted into treatment for both sick individuals and those that they infect.

CHAPTER 7

7. SUMMARY AND RECOMMENDATION

7.1 Summary

This study was conducted by Central Department of Microbiology, Tribhuvan University in collaboration with National Tuberculosis Centre during September 2005 to May 2006 to study the anti-TB drugs resistance pattern in PTB patients.

Among 295 PTB cases included in the study, 73.56% were male and 26.44% were female. Out of 295 PTB cases, 250 cases were previously treated cases (old cases) and 45 were untreated cases (new cases). Antibiotic susceptibility test of culture positive isolates were performed in the first line anti-TB drugs INH, SM, RMP and EMB by Proportion method. EMB (66.10%) was found to be the most effective anti-TB drug according to the susceptibility test followed by RMP (60.33%), SM (59.66%) and INH (41.69%) against *M. tuberculosis*.

Among 45 isolates isolated from untreated patients, primary drug resistance to one drug was in 20%, to two drugs in 17.77%, to three drugs in 11.11%, to four drugs in 6.66% and primary Multi-drug resistant in 22.22% of the isolates.

Among 250 isolates isolated from previously treated patients, acquired resistance to one drug was found in 23.60%,to two drugs in 12.40%, to three drugs in 16.40%, to four drugs in 18.80% an acquired Multi-drug resistant in 37.20% of the isolates.

Among 250 treated cases,68.40%(n=171) were relapse,18%(n=45) were chronic,7.6%(n=19) were follow-up,3.2%(n=8)were defaulter, and 2.8%(n=7) were treatment failure. MDR-TB was found the highest in chronic cases (64.44%) followed by follow-up case (47.36%), treatment failure cases (42.85%), relapse cases (27.48%) and Default cases (12.5%).

A statistical analysis reveals no significant relationship between age and sex with the emergence of drug resistant isolates. Also there was no relationship between drug resistance with habit of smoking, taking alcohol and family history of TB.

7.2 Recommendations

1. Drug resistant surveillance should be frequently done to document the level of drug resistance in the community in order to monitor the impact of the program over time and also to ensure the appropriate treatment regimens.

2. Regular monitoring of MDR-TB and policy planning should be based on the operational researches and findings help the control.

3. Standardized recording and reporting on all categories of re-treatment cases like chronic, relapse, treatment failure, and return after default and follow-up should be continued. Accurate reporting on this population will help in the monitoring performance and developing re-treat strategies.

4. The MDR isolates can be further analyzed utilizing epidemiological typing including biotyping, phagetyping, serotyping, DNA profiling (RFLP) which may open doors to the epidemiological as well as the genetic aspects of MDR.

CHAPTER 8

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APPENDICES

Appendix I: Materials

Bacteriological media

LJ medium

2% Ogawa medium

B. Reagents/chemicals

Absolute alcohol

Carbol Fuchsin

Spirit

Phenol

Methylene blue

Ethanol

Sodium hydroxide

Distilled water

Egg

Glycerol Merck

Malachite green

Cidar wood oil

Sulphuric acid

C. Glasswares

Beaker

Conical flask

Measuring cylinder

Test tubes

Culture tubes

Glass rods

Pasteur pipette

Pipette

Micropipette

Slides

Petri plates

Glass funnel

Diamond pencil

D. Equipments

Microscope	Olympus, Japan
Centrifuge	Kuboto, Japan
Biological Safety Cabinet	Kuboto, Japan
Autoclave	Kuboto, Japan
Incubator	IKemoto, Japan
Refrigerator	Sanyo, Japan
Coagulator	Hirasawa, Japan
Hot air oven	IKemoto, Japan
Fluorescent microscope	Olympus, Japan
Dryer	Philips
Vortex mixer	Remi, equipment, India
Electronic balance	Kuboto, Japan
Inspissator	Chemical and instrumentals corpor.India
Distilling apparatus Blender	Sanyo, Japan

E. Pipettes and tubes

Eppendrof micropipette

Fine pipette

Micropipette tip

F. Miscellaneous

Bacteriological loop

Cotton

Forceps

Gloves

Staining rack

Spirit lamp

Soaps

Tube holder

Tissue paper

Sputum container

Bamboo stick

Bunsen burner

Appendix II: Bacteriological Media

Composition and preparation of culture media

1. Salt solution Lowenstein-Jensen media (Ingredients)

Potassium dihydrogen phosphate	=	2.0 gm
Magnesium sulphate	=	0.2 gm
Trimagnesium dicitrate	=	0.5 gm
L-Asparagine	=	3.0 gm
Distilled water	=	500.0 ml.

Weighed individually and dissolved one by one with the help of hot water (water bath) and autoclaved at 121^0C for 15 minutes.

2. Glycerine	=	10 ml
Starch	=	25 gm

3. Malachite green 2%

Malachite green dye	=	2.0 gm
Distilled water	=	100 ml

4. Penicillin solution	=	0.5 ml

5. Homogenized whole eggs

Fresh hen's were cleaned by brush and soap and then run with tap water, then dried and wiped the outer surface with spirit. The eggs were cracked with a sterile knife into a sterile beaker and eggs were homogenized with magnetic stirrer.

Preparation of medium

About 10 ml of glycerine was added in 500 ml salt solution and mixed then and added 25 gm of starch till the solution become a homogenous by the help of hot water bath (90-85^{o}C/90 minutes). About 830 ml of egg mass were added and mixed well and then finally added 13.5 ml of 2% malachite green and 0.5 ml of penicillin solution (for

inhibition of contaminants) and mixed well then filled in the culture tubes about 5ml . The mixture in the tubes was then heated at 85^{o}C for 95 minutes in hot water bath in slantwise position. The tubes were cooled and stored in refrigerator.

2% Modified Ogawa Medium

1. Preparation of salt solution	500ml/flask
Potassium dihydrogen phosphate	2.0 gm
Magnesium citrate	0.1 gm
Sodium Glutamate	0.5 gm
D/W	100 ml

- Mix well and heat at 100°C for 30 minutes in a water bath (or autoclave at 121°C for 15 minutes).
- Add glycerol 4ml into the salt solution (while it is hot).
- Add 4ml 2% malachite green solution.

2. Preparation of whole egg homogenate

- Wipe off eggshell with spirit cotton after washing it with brush and detergent.
- Break down the egg to a Petri-plate to check the decomposition
- Transfer the egg into a beaker (500 ml)
- Homogenize the eggs in a mixer-grinder until the eggs become watery.
- Place the two layers of sterile gauze pieces on the funnel
- Filter the egg homogenate to get 200 ml.

3. Mix 1 with 2 (Raw Modified Ogawa Medium)

4. Distribution of raw medium

- Dispense the medium 6ml into each tube (avoid bubble formation)

5. Inspissations

- Arrange the tubers in slant position and coagulate them at 90°C for one hour with caps closely closed

6. Store at 4°C - 6°C with caps closed tightly.

Coagulation of medium

Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C (Since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilize it). Heating for a second or third time has a detrimental effect on the quality of the medium.

The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures.

Poor quality media should be discarded.

- Sterility Check

After inspissations, the whole media batch or a representative sample of culture bottles should be incubated at 35°-37°C for 24 hours as a check of sterility.

- Storage

The LJ medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than 4 weeks.

For the cultivation of *M. bovis,* LJ medium is enriched with 0, 5% sodium pyruvate. Glycerol is omitted and 8.0g sodium pyruvate is added to the mineral solution.

Appendix III: Reagents and chemicals

Composition and preparation of staining reagents

Fluorescence staining reagents

i. Auramine solution

Auramine	=	1.0 gm
Ethanol	=	100.0 ml
Phenol	=	30.0 ml
Distilled water	=	870.0 ml

Weighed auramin was dissolved in ethanol and phenol liquid in water and mixed both solution and kept in dark bottle.

ii. 20% Sulphuric acid (Decolorizer)

Sulphuric acid (conc.)	=	200.0 ml
Distilled water	=	800.0 ml

Always add sulphuric acid in water.

iii. Methylene Blue (Counter stain)

Methylene blue	=	1.0 gm
Distilled water	=	1000.0 ml

About 1 gm of methylene blue was added in 1000 ml distilled water and mixed well.

Digestion and decontamination regents

i.Nekal solution

Nekal	=	0.1 gm
Sodium hydroxide	=	5.0 gm
Sodium triphosphate (Na_3PO_4)	=	1.0 gm

Distilled water	=	1000 ml

ii. Calcium chloride + Barium chloride solution

Calcium chloride ($CaCl_2$)	=	2.0 gm
Barium chloride ($BaCl_2$)	=	4.0 gm
Distilled water	=	100.0 ml

Composition and preparation of different biochemical reagents and media

1. Niacin buffer

Sodium nitrate ($NaNO_3$)	=	0.85 gm
KH_2PO_4	=	1.17 gm
Na_2HPO_4	=	4.85 gm/1
Distilled water	=	1000 ml

These all constituents were prepared and adjusted the pH 7.

1. Catalase reagents

Tween 80 (10%)

Tween 80	=	10 ml
Distilled water	=	90 ml

About 10 ml of Tween 80 was added to 90 ml of distilled water and mixed well. The mixture was autoclaved at 121^0C for 10 minutes. The Tween settle during autoclaving. It was resuspended by swirling.

Complete catalase reagent

Equal parts of 10% tween 80 and 30% of Hydrogen peroxide were mixed well to produce tween peroxide mixture a complete catalase regent.

2. 25% Hydrochloric acid solution: (1:2 dilution)

Conc. HCl	=	5 ml
Distilled water	=	5 ml

3. 0.2 Sulfanilamide solution

Sulfanilamide	=	0.2 gm
Distilled water	=	100 ml

About 0.2 gm sulfanilamide was dissolved in 100 ml of distilled water and stored in bottle.

5. 0.1% N-Naphthyl-ethylene-diamine-dihydrochloride

N-Naphthyl-ethylene-diamine-dihydrochloride	=	0.1 gm
Distilled water	=	100 ml.

Preparation of drugs solution and drugs containing media for drug sensitivity

Rifampicin (RMP)

1. Phosphate buffer: 174.3 mg KH_2PO_4 and 959.6 mg Na_2HPO_4 were mixed in 100 ml Distilled water

Solution I: 27.17 mg RMP was mixed in 6.5 ml distilled water (4000 µg/ml)

Solution II: 4 ml Sol. I add up to 40 ml with phosphate buffer (400 µg/ml)

Concentration in drug media (µg/ml)

	40 µg/ml	20 µg/ml
Media (ml)	135	135
Aqua distilled water	---	9
Sol II	15	7.5
Total	150 ml	150 ml

1. Streptomycin (SM)

Solution I: 27 mg SM mix in 4 ml distilled water (5000 µg/ml)

Solution II: 1 ml Sol. I add up to 50 ml distilled water (100 µg/ml)

Concentration in drug media (µg/ml)

	8 µg/ml	4 µg/ml
Media (ml)	135	135
Aqua distilled water	3	9
Sol II	12	6
Total	150 ml	150 ml

1. Isoniazid (INH)

Solution I: 50 mg INH mix with 10 ml distilled water (5000 µg/ml)

Solution II: 1 ml Sol. I add up to 50 ml distilled water (100 µg/ml)

Solution III: 2.5 ml Sol. I add up to 25 ml distilled water (10 µg/ml)

Concentration in drug media (µg/ml)

	8 µg/ml	0.25 µg/ml
Media (ml)	135	135
Aqua distilled water	3	11.25
Sol II	12	—
Sol III	—	3.75
Total	150 ml	150ml

4. Ethambutol (EMB)

Solution I: 4 mg EMB mix in 25 ml distilled water (1000 µg/ml)

Solution II 1 ml Sol. I add up to 50 ml distilled water (20 µg/ml)

Concentration in drug media (µg/ml)

	2 µg/ml	1 µg/ml
Media (ml)	135	135
Aqua distilled water	—	7.5
Sol. II	15	7.5
Total	150 ml	150 ml

Appendix IV: Questionnaire for the PTB patients

Lab No.

A. Identification of the patient:

i) Name:…… ii) Age/Sex……M/F

iii) Education: iv) Occupation:

B. Address of the patient:

i) Zone: ii) District:

iii) VDCP/ Municipality: iv) Ward No:

v) Village /City:

C. Habit:

i) Smoking: Yes/ No: ii) Alcoholic: Yes/ No

D. Family history of the patient:

i) Previous case of TB in his/her family: Yes/ No

ii) Relation:

E. History given by the patient:

Previously treated for TB: Yes/ No.

If the answer is 'No', go to E2 but if the answer is 'Yes' go to E3.

E2 Standardized history:

i) How long have you been sick?

ii) Did you have the same symptoms prior to this episode?

iii) Did you have other symptoms of lung diseases prior to this episode (Haemoptysis, chest pain, cough)?

iv) Did you have X-ray examination prior to the episode?

v) Did you have sputum examinations prior to this episode?

vi) Did you ever take tuberculosis drugs for more than one month?

If Yes, what were the names of the drugs?

vii) Did you ever have injections for more than one month?

Did the patient remember pervious treatment for TB after these questions?

Yes/ No

If Yes, continue with E3.

E3.Information about previous treatment:

i) Where was the patient treated?

ii) When was the patient treated?

iii) How many times was the patient treated?

iv) Which drugs were used for treatment?

Category I/Category II/ Category III/ Others.

v) How many courses of treatment were given?

vi) Outcome of the last treatment according to the patient:

Cured/Not cured/Unknown

Appendix V: Master chart

Results of 295 samples showing sensitivity pattern test of *Mycobacterium tuberculosis* on LJ Medium

SN.	SAMPLE NO.	AGE	SEX	TYPE	CONTROLS	INH		SM		RMP		EMB		REMARKS
						0.25ug/ml	8ug/ml	4ug/ml	8ug/ml	20ug/ml	40ug/ml	1ug/ml	2ug/ml	
1	567	35	M	TF	3+2+1+	2+	-	-	-	-	-	-	-	H
2	468	31	M	TF	3+2+1+	3+	-	3+	2+	3+	2+	2+	1+	HRSE
3	508	61	M	RAD	3+2+1+	1+	-	2+	1+	-	-	1+	-	HS
4	509	19	F	Re	3+2+1+	1+	-	2+	1+	-	-	1+	-	HS
5	526	39	F	Chr	3+2+1+	-	-	1+	-	-	-	-	-	S
6	528	32	M	Re	3+2+1+	2+	-	2+	1+	3+	2+	2+	1+	HRSE
7	529	26	M	Re	3+2+1+	1+	-	2+	1+	2+	1+	1+	1+	HRSE
8	531	24	M	Chr	3+2+1+	1+	-	3+	2+	3+	2+	3+	2+	HRSE
9	533	39	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
10	575	39	M	Chr	3+2+1+	3+	3+	-	-	-	-	3+	3+	HE
11	580	62	M	FU	3+2+1+	-	-	-	-	-	-	-	-	-
12	534	38	M	Re	3+2+1+	-	-	3+	2+	-	-	-	-	S
13	536	20	F	Re	3+2+1+	-	-	-	-	-	-	1+		-

14	537	70	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
15	541	67	M	N	3+2+1+	-	-	-	-	-	-	-	-	-
16	542	30	M	Re	3+2+1+	+	-	-	-	-	-	-	-	H
17	543	58	M	Re	3+2+1+	1	-	-	-	-	-	-	-	-
18	563	68	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
19	570	35	M	Chr	3+2+1+	-	-	2+	1+	3+	1+	1+	-	HRS
20	571	21	M	Re	3+2+1+	3+	-	-	-	-	-	-	-	-
21	572	39	M	Chr	3+2+1+	-	-	-	-	-	-	-	-	-
22	485	60	M	FU	3+2+1+	-	-	2+	1+	2+	1+	2+	2+	HRSE
23	511	60	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	-
24	512	17	F	Re	3+2+1+	-	-	-	-	-	-	-	-	-
25	569	66	F	FU	3+2+1+	-	-	-	-	-	2+	2+	-	-
26	579	44	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
27	581	51	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
28	582	20	F	Chr	3+2+1+	-	-	-	-	-	-	-	-	-
29	2	28	M	Re	3+2+1+	1+	-	-	1+	-	-	-	-	H
30	7	37	M	Re	3+2+1+	1+	-	2+	-	2+	1+	-	-	HRS
31	6	36	M	N	3+2+1+	-	-	-	-	-	-	-	-	-
32	3	42	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-

33	8	30	M	RAD	3+2+1+	-	-	-	-	-	-	-	-	-
34	10	42	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
35	13	18	M	Re	3+2+1+	-	-	-	-	-	-	-	-	H
36	15	31	F	Re	3+2+1+	3+	-	-	-	2+	-	-	-	H
37	16	45	M	N	3+2+1+	3+	2+	-	1+	-	-	-	-	RS
38	17	62	M	Re	3+2+1+	-	1+	2+	-	3+	2+	-	-	HR
39	14	70	F	Re	3+2+1+	2+	-	-	1+	3+	2+	1+	-	HRS
40	20	50	M	Chr	3+2+1+	3+	-	2+	-	2+	1+	1+	-	-
41	22	27	M	Re	3+2+1+	-	-	-	1+	-	-	-	-	HRS
42	23	19	M	TF	3+2+1+	2+	-	2+	+	2+	1+	1+	-	HRSE
43	25	34	M	Re	3+2+1+	3+	-	3+	2+	3+	2+	3+	2+	-
44	26	30	M	N	3+2+1+	-	-	-	-	-	-	-	-	-
45	27	50	M	N	3+2+1+	-	-	-	-	-	-	-	-	HRSE
46	30	28	M	Chr	3+2+1+	3+	-	2+	1+	3+	1+	2+	1+	HRSE
47	31	48	F	Re	3+2+1+	3+	-	2+	1+	3+	2+	3+	2+	HRSE
48	32	35	F	N	3+2+1+	2+	-	2+	1+	2+	1+	2+	1+	HRSE
49	33	61	M	Chr	3+2+1+	1+	-	2+	1+	2+	1+	2+	1+	HRSE
50	35	23	F	Re	3+2+1+	2+	-	-	-	2+	1+	2+	1+	HRE
51	37	48	M	Re	3+2+1+	-	-	-	-	-	-	2+	-	-

52	40	29	M	Re	3+2+1+	-	-	-	-	-	-	2+	2+	E
53	36	32	F	RAD	3+2+1+	-	-	-	-	-	-	-	-	-
54	1024(A)	20	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
55	282(A)	35	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
56	1020(A)	29	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
57	778(A)	41	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
58	371(A)	60	F	N	3+2+1+	-	-	2+	-	-	-	-	-	S
59	702(A)	18	F	N	3+2+1+	3+	-	-	-	-	-	-	-	
60	412	25	M	Re	3+2+1+	3+	-	3+	3+	3+	3+	3+	-	HRSE
61	415	33	M	Re	3+2+1+	1+	-	-	-	-	-	2+	-	H
62	417	26	M	Re	3+2+1+	-	-	3+	-	1+	-	-	-	S
63	418	25	M	Re	3+2+1+	2+	-	2+	1+	2+	**1+**	2+	1+	HRSE
64	420	32	M	Re	3+2+1+	-	-	2+	2+	-	-	-	-	S
65	425	34	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	-
66	413	17	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
67	422	30	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
68	423	19	F	Re	3+2+1+	-	-	-	-	-	-	-	-	-
69	RT6	71	M	N	3+2+1+	1+	1+	1+	1+	1+	**1+**	1+	1+	HRSE
70	426	33	M	Re	3+2+1+	-	-	2+	-	-	3+	3+	1+	S

71	427	55	M	Re	3+2+1+	3+	-	1+	-	3+	-	1+	-	HR
72	429	56	M	Re	3+2+1+	-	-	-	-	3+	-	1+	-	-
73	430	37	M	Chr	3+2+1+	-	-	-	3+	3+	3+	3+	3+	-
74	431	22	F	N	3+2+1+	-	-	-	-	-	2+	3+	3+	Re
75	432	22	F	N	3+2+1+	3+	3+	-	-	-	2+	3+	3+	HRE
76	435	39	M	Chr	3+2+1+	3+	3+	-	-	3+	-	-	-	HR
77	418	26	M	Re	3+2+1+	-		-	-	3+	-	-	-	-
78	439	22	M	N	3+2+1+	3+		3+	-	3+	3+	-	-	HS
79	440	17	F	Chr	3+2+1+	2+		-	3+	-	3+	3+	3+	HR
80	441	19	F	Chr	3+2+1+	3+		3+	2+	-	2+	2+	1+	HR
81	442	26	M	RAD	3+2+1+	2+		3+	-	2+	-	-	-	HR
82	443	16	M	Re	3+2+1+	-		-	-	-	-	-	-	-
83	444	68	M	Re	3+2+1+	-		-	-	2+	-	3+	3+	-
84	447	35	M	Re	3+2+1++	3+		-	-	-	-	-	-	HE
85	448	54	M	Re	3+2+1+	-		2+	-	-	2+	2+	-	-
86	450	22	F	N	3+2+1+	2+		-	2+	-	-	-	-	HR
87	454	42	M	FU	3+2+1+	-		-	-	2+	-	2+	-	-
88	455	50	F	RAD	3+2+1+	-		2+	2+	3+	2+	-	-	-
89	456	41	M	Re	3+2+1+	2+		-	-	-	-	2+	1+	HRS

90	457	35	M	Re	3+2+1+	-		3+	2+	-	2+	-	-	-
91	459	35	F	Re	3+2+1+	2+		2+	-	-	-	-	1+	H
92	461	22		N	3+2+1+	-		1+	-	3+	-	-	-	S
93	462	45	M	N	3+2+1+	1+	-	-	-	-	-	-	-	H
94	463	45	M	Chr	3+2+1+	-	-	-	-	-	-	2+	1+	H
95	470	34	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
96	471	60	M	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
97	473	50	M	Re	3+2+1+	1+	-	-	-	3+	-	-	-	H
98	476	25	F	Chr	3+2+1+	3+	-	1+	-	-	3+	2+	1+	HRS
99	481	45	M	N	3+2+1+	-	-	-	-	-	-	-	-	-
100	484	45	M	Re	3+2+1+	-	-	-	-	-	-	-	1+	-
101	493	32	M	N	3+2+1+	-	-	-	-	-	-	-	1+	-
102	501	22	M	Re	3+2+1+	-	-	1+	-	1+	-	-	1+	-
103	506	26	M	Re	3+2+1+	1+	-	-	1+	3+	1+	2+	1+	HRSE
104	510	28	M	Re	3+2+1+	3+	1+	2+	2+	-	3+	1+	-	HRS
105	515	32	F	Re	3+2+1+	1+	-	-	-	-	-	2+	-	H
106	514	41	F	Re	3+2+1+	1+	-	-	-	2+	-	-	-	H
107	502	25	F	Re	3+2+1+	1+	-	2+	1+	3+	2+	3+	-	HRSE
108	505	22	F	Re	3+2+1+	-	-	-	-	2+	-	-	-	-

109	511	61	M	Re	3+2+1+	2+	1+	2+	1+	-	2+	2+	1+	HRSE
110	520	25	M	FU	3+2+1+	3+	2+	3+	1+	-	1+	3+	1+	HRSE
111	521	48	F	Re	3+2+1+	2+	2+	2+	1+	2+	2+	2+	1+	HRSE
112	522	27	M	N	3+2+1+	-	-	-	-	-	1+	2+	1+	RE
113	523	48	M	Re	3+2+1+	-	-	-	-	3+	-	-	-	-
114	525	22	M	Re	3+2+1+	-	-	1+	-	-	-	-	-	S
115	557	43	M	Re	3+2+1+	-	-	1+	-	2+	1+	1+	-	RS
116	558	26	M	RAD	3+2+1+	2+	-	-	-	-	-	-	-	H
117	559	24	M	Re	3+2+1+	2+	-	2+	1+	3+	2+	1+	-	HRS
118	562	36	M	TF	3+2+1+	-	-	-	-	-	-	-	-	-
119	45	30	F	Re	3+2+1+	-	-	-	-	-	-	-	2+	E
120	46	28	F	Chr.	3+2+1+	1+	-	2+	1+	2+	1+	2+	1+	HRES
121	50	20	M	FU	3+2+1+	2+	1+	2+	1+	2+	1+	2+	1+	HRES
122	54	40	M	Re	3+2+1+	1+	-	-	-	-	-	2+	1+	HE
123	63	64	M	Chr.	3+2+1+	2+	-	2+	1+	2+	1+	2+	1+	HERS
124	66	38	F	Chr.	3+2+1+	2+	1+	3+	2+	3+	2+	3+	2+	HERS
125	72	16	M	Chr.	3+2+1+	2+	-	3+	2+	3+	2+	3+	2+	HRES
126	75	50	M	Re	3+2+1+	2+	-	-	-	-	-	2+	1+	HE
127	70	44	M	Chr.	3+2+1+	3+	2+	-	-	3+	2+	2+	1+	HRE

128	76	50	M	Re	3+2+1+	2+	1+	1+	-	3+	2+	2+	1+	HRES
129	79	45	M	Re	3+2+1+	-	-	-	-	-	-	2+	-	E
130	80	68	F	Chr.	3+2+1+	-	-	-	-	-	-	-	-	-
131	82	24	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
132	84	27	M	Chr.	3+2+1+	-	-	-	-	-	-	2+	1+	E
133	85	45	M	FU	3+2+1+	1+	-	2+	1+	2+	1+	3+	2+	HRSE
134	87	61	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	H
135	88	62	M	Re	3+2+1+	-	-	-	-	-	-	2+	-	-
136	93	52	M	Re	3+2+1+	2+	-	2+	1+	-	-	-	-	HS
137	95	31	M	Re	3+2+1+	2+	-	-	-	-	-	2+	-	H
138	96	55	M	Re	3+2+1+	3+	1+	1+	-	2+	1+	2+	1+	HRSE
139	58	25	M	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
140	71	15	M	N	3+2+1+	2+	1+	2+	1+	2+	1+	2+	1+	HRSE
141	64	54	M	Chr.	3+2+1+	-	-	-	-	-	-	-	-	-
142	78	42	M	Re	3+2+1+	2+	-	-	-	2+	1+	2+	1+	HRE
143	101	21	M	Chr	3+2+1+	2+	-	-	-	3+	2+	3+	1+	HRE
144	102	42	F	Re	3+2+1+	2+	1+	2+	1+	3+	2+	2+	1+	HRES
145	103	27	M	Re	3+2+1+	3+	1+	3+	2+	3+	2+	3+	2+	HRES
146	105	43	M	Re	3+2+1+	-	-	-	-	-	-	2+	-	-

147	106	44	M	N	3+2+1+	2+	-	-	-	-	-	2+	-	H
148	107	88	M	Re	3+2+1+	3+	**2+**	3+	2+	3+	2+	3+	2+	HRES
149	97	37	F	Re	3+2+1+	3+	2+	-	-	-	-	2+	1+	HE
150	108	42	F	Re	3+2+1+	2+	1+	-	-	-	-	3+	2+	HE
151	109	17	M	Re	3+2+1+	2+	1+	-	-	2+	1+	3+	2+	HRE
152	110	27	M	Chr	3+2+1+	2+	1+	-	-	-	-	-	-	H
153	111	26	M	Chr	3+2+1+	1+	-	2+	1+	1+	-	2+	1+	HSE
154	112	50	M	N	3+2+1+	2+	1+	-	-	-	-	2+	1+	HE
155	113	35	F	Chr	3+2+1+	2+	1+	3+	2+	3+	2+	-	-	HRS
156	115	75	M	Re	3+2+1+	2+	-	-	-	-	-	3+	2+	HE
157	119	61	M	Re	3+2+1+	3+	1+	-	-	-	-	2+	1+	HE
158	120	20	M	Re	3+2+1+	2+	-	-	-	-	-	2+	1+	HE
159	121	50	M	Re	3+2+1+	2+	1+	-	-	-	-	3+	2+	HE
160	123	36	M	N	3+2+1+	3+	2+	-	-	-	-	3+	2+	HE
161	42	65	M	TF	3+2+1+	3+	2+	1+	-	2+	1+	1+	-	HR
162	43	15	F	Re	3+2+1+	1+	-	2+	1+	2+	1+	2+	1+	HRES
163	53	35	M	Re	3+2+1+	2+	-	1+	-	3+	1+	1+	-	HRS
164	54	18	F	O/N	3+2+1+	1+	-	2+	1+	2+	1+	1+	-	HRS
165	56	79	M	O/N	3+2+1+	2+	-	-	-	-	-	1+	-	H

166	53	37	M	FU	3+2+1+	1+	-	-	-	-	-	1+	-	H
167	74	26	M	FU	3+2+1+	-	-	-	-	-	-	-	-	-
168	117	49	M	RE	3+2+1+	1+	-	-	-	-	-	2+	-	H
169	118	18	M	Chr	3+2+1+	2+	-	1+	-	1+		2+	1+	HSE
170	126	74	M	Re	3+2+1+	3+	2+	3+	2+	3+	2+	3+	2+	HRES
171	129	24	F	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
172	132	43	M	Re	3+2+1+	2+	1+	2+	1+	1+	1+	1+		HRS
173	136	19	M	Re	3+2+1+	2+	1+	1+	-	1+	-	2+	1+	HSE
174	137	65	M	Re	3+2+1+	2+	1+	-	-	-	-	-	-	H
175	138	52	M	Re	3+2+1+	3+	2+	-	-	-	-	2+	1+	HE
176	125	45	M	Chr	3+2+1+	1+	-	-	-	2+	1+	2+	1+	HRE
177	128	67	M	FU	3+2+1+	1+	1+	-	-	-	-	1+	1+	H
178	150	29	M	Re	3+2+1+	2+	-	-	-	-	-	2+	-	HE
179	131	34	M	Re	3+2+1+	-	-	-	-	-	-	1+	-	H
180	133	43	M	N	3+2+1+	2+	1+	-	-	-	-	1+	-	-
181	140	15	M	N	3+2+1+	-	-	-	-	-	-	2+	-	-
182	143	62	M	FU	3+2+1+	1+	-	2+	1+	2+	1+	3+	2+	HRES
183	144	30	M	Re	3+2+1+	2+	1+	-	-	2+	1+	-	-	HR
184	145	26	M	Re	3+2+1+	2+	-	-	-	-	-	2+	1+	HE

185	147	37	F	Re	3+2+1+		-	-	-	-	-	1+	-	-
186	127	62	F	N	3+2+1+		-	3+	2+	-	-	1+	-	S
187	142		M	Re	3+2+1+		-	-	-	2+	1+	2+	1+	Re
188	150	44	M	Re	3+2+1+	3+	2+			3+	2+	1+	-	HR
189	152	18	F	Re	3+2+1+	-	-	3+	2+	3+	2+	3+	2+	RSE
190	153	65	M	Re	3+2+1+	-	-	-	-	-	-	3+	2+	E
191	155	39	F	Re	3+2+1+	-	-	3+	2+	3+	2+	1+		HR
192	156	25	F	Re	3+2+1+	-	-					2+	1+	E
193	159	37	M	N	3+2+1+	-	-	3+	2+	3+	2+	1+	-	RS
194	161	44	M	Re	3+2+1+	-	-	-	-	-	-	3+	2+	E
195	162	27	M	Re	3+2+1+	-	-	-	-	3+	2+	-	-	R
196	164	32	M	Re	3+2+1+	-	-	-	-	-	-	2+	1+	E
197	169	52	M	Re	3+2+1+	2+	2+	3+	2+	-	-	2+	1+	HSE
198	148	20	F	N	3+2+1+	-	-	3+	2+	3+	2+	-	-	SR
199	157	11	M	Chr	3+2+1+	3+	2+	3+	2+	3+	2+	-	-	HRS
200	163	28	M	Re	3+2+1+	2+	-	3+	2+	3+	2+	-	-	HRS
201	170	58	M	N	3+2+1+	1+	-	-	-	-	-	-	-	H
202	171	60	F	N	3+2+1+	-	-	-	-	-	-	-	-	-
203	172	22	F	Chr	3+2+1+	1+	-	3+	2+	3+	2+	2+	1+	HRSE

204	174	20	M	Re	3+2+1+	3+	-	3+	2+	3+	2+	-	-	HRS
205	176	19	F	N	3+2+1+	-	-	3+	2+	3+	2+	-	-	SR
206	177	37	M	RAD	3+2+1+	-	-	-	-	-	-	-	-	-
207	179	19	M	Re	3+2+1+	1+	-	-	-	-	-	1+	-	H
208	180	45	M	Re	3+2+1+	-	-	-	-	-	-	2+	1+	E
209	165	18	M	Chr	3+2+1+	1+	-	3+	2+	3+	2+	-	-	HRS
210	166	35	F	Chr	3+2+1+	2+	1+	-	-	3+	2+	-	-	HR
211	173	50	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
212	175	71	M	Re	3+2+1+	2+	1+	2+	-	1+	-	-	-	HRS
213	181	32	F	Re	3+2+1+	-	-	-	-	2+	-	-	-	R
214	182	30	F	Re	3+2+1+	-	-	-	-	-	-	-	-	-
215	183	22	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	H
216	184	51	M	Re	3+2+1+	1+		3+	2+	2+	1+	1+		HRS
217	188	42	M	Re	3+2+1+	2+	1+	-	-		2+	2+		HE
218	190	23	F	Re	3+2+1+	1+	-	-	-	2+	2+	2+	1+	HRE
219	195	46	M	Re	3+2+1+	-	-	-	-	-	-	-	1+	-
220	196	63	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
221	59	33	M	Re	3+2+1+	3+	2+	-	-	2+	2+	2+	-	HRE
222	61	16	M	Re	3+2+1+	1+	-	3+	2+	2+	-	-	1+	HRS

223	63	21	M	Re	3+2+1+	3+	-	3+	2+	2+	2+	2+	-	HRSE
224	186	44	F	N	3+2+1+	3+	1+	2+		2+	-	-	1+	HRS
225	199	33	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	H
226	200	27	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
227	202	40	M	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
228	204	42	M	Chr	3+2+1+	2+	1+	-	-	1+	2+	2+	-	HRE
229	187	60	F	Re	3+2+1+			3+	-	-	-	-	1+	S
230	206	30	M	Chr	3+2+1+	2+	1+	3+	3+	3+	2+	2+	-	HRSE
231	208	35	F	Re	3+2+1+			3+			2+	2+	1+	S
232	209	42	M	Re	3+2+1+	3+	1+	3+	2+	3+	3+	3+	2+	HRSE
233	210	42	M	Re	3+2+1+	3+	-	3+	3+	-	2+	2+	-	HS
234	211	46	M	Re	3+2+1+	3+	-	3+	3+	3+	3+	3+	-	HRS
235	212	24	M	RAD	3+2+1+	-	-	1+	-	-	-	2+	-	S
236	213	24	M	Re	3+2+1+	-	-	3+	2+	-	2+	1+	-	S
237	214	23	M	Chr	3+2+1+	3+	-	3+	1+	3+	-	3+	2+	HRSE
238	215	45	M	Chr	3+2+1+	-	-	3+	-	-	-	2+	-	S
239	216	19	F	Re	3+2+1+	-	-	3+	-	-	-	1+	-	S
240	223	32	F	Re	3+2+1+	3+	-	3+	2+		2+	3+	-	S
241	224	38	M	Re	3+2+1+	-	2+	3+	2+	3+	2+	3+	2+	HRSE

242	227	21	F	Chr	3+2+1+	3+	-	3+	2+	3+	2+	3+	2+	HRSE
243	228	37	M	Chr	3+2+1+	3+	2+	3+	2+	3+	-	3+	-	HRS
244	233	22	M	Re	3+2+1+	-	-	3+	-	-	2+	1+	-	S
245	193	38	M	Re	3+2+1+	-	-	1+	-	2+	-	2+	-	SR
246	201	34	F	Re	3+2+1+	-	-	2+	-	-	1+	1+	-	S
247	229	26	M	Chr	3+2+1+	-	-	1+	-	2+	-	1+	-	SR
248	230	58	M	Re	3+2+1+	-	-	2+	-	-	2+	2+	-	S
249	231	20	F	FU	3+2+1+	2+	-	2+	2+	2+	2+	2+	-	HRS
250	232	68	F	Chr	3+2+1+	2+	-	2+	2+	2+	3+	2+	-	HRS
251	234	28	M	Chr	3+2+1+	3+	-	2+	-	3+	-	2+	-	HRS
252	237	28	M	Re	3+2+1+	-	-	-	-	-	-	1+	-	-
253	219	47	M	N	3+2+1+	-	-	-	-	-	-	-	-	-
254	236	26	F	Re	3+2+1+	3+	-	-	-	-	-	-	-	H
255	238	23	M	Re	3+2+1+	3+	-	-	-	-	-	-	-	H
256	239	48	M	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
257	240	24	M	Re	3+2+1+	1+	-	1+	-	1+	-	1+	-	HS
258	242	58	M	Re	3+2+1+	1+	-	-	-	3+	3+	-	-	HR
259	243	72	M	Re	3+2+1+	2+	-	-	-	-	-	-	-	H
260	245	38	M	Re	3+2+1+	1+	-	2+	2+	2+	-	3+	2+	HSE
261	246	62	M	Re	3+2+1+	2+	-	2+	2+	2+	2+	-	-	HRS

262	249	50	F	Re	3+2+1+	1+	-	-	-	1+	-	-	-	H
263	250	51	M	Chr	3+2+1+	1+	-	-	-	1+	-	1+	-	H
264	252	17	M	Re	3+2+1+	2+	-	3+	3+	3+	3+	3+	3+	HRSE
265	253	26	M	Chr	3+2+1+	3+	-	3+	3+	3+	3+	-	-	HRS
266	258	34	M	Re	3+2+1+	1+	-	-	-	-	-	-	-	H
267	259	21	M	Re	3+2+1+	3+	-	3+	3+	3+	3+	3+	3+	HRSE
268	248	25	M	Re	3+2+1+		-	-	-	-	-	3+	1+	E
269	256	26	M	Re	3+2+1+	3+	1+	-	-	2+	1+	2+	1+	HRE
270	260	52	M	Chr	3+2+1+	3+	-	-	-	3+	3+	3+	3+	HRE
271	262	29	M	Re	3+2+1+	-	-	-	-	-	-	-	-	-
272	263	53	M	Re	3+2+1+	-	-	-	-	2+	-	-	-	-
273	265	37	M	Re	3+2+1+	3+	-	-	-	-	-	-	-	H
274	274	22	M	Re	3+2+1+		-	-	-	-	-	-	-	-
275	275	61	M	Re	3+2+1+	3+	-	-	-	-	-	-	-	H
276	276	55	F	FU	3+2+1+	-	-	-	-	-	-	-	-	-
277	277	28	M	FU	3+2+1+	-	3+	-	-	3+	3+	3+	3+	RE
278	278	56	M	Re	3+2+1+	-	-	-	-	-	-	3+	-	-
279	64	36	M	FU	3+2+1+	3+	3+	3+	3+	3+	3+	3+	3+	HRSE
280	65	25	M	N	3+2+1+		-	-	-	-	-	3+	-	-
281	68	22	M	N	3+2+1+		-	-	-	2+	-	-	-	-

282	67	22	M	N	3+2+1+	3+	-	-	-	3+	3+	-	-	HR
283	68	67	M	N	3+2+1+	1+	-	-	-	3+	3+	2+	1+	HRE
284	70	27	M	N	3+2+1+	1+	-	-	-	1+	-	-	-	H
285	264	60	M	TF	3+2+1+	-	-	-	-	-	-	2+	-	-
286	271	40	M	FU	3+2+1+	2+	-	-	-	3+	3+	2+	2Col	HR
287	272	20	F	TF	3+2+1+	1+	-	-	-	1+	1+	1+	-	HRS
288	281	52	F	Re	3+2+1+	-	-	2+	-	-	-	-	-	-
289	282	24	F	FU	3+2+1+	3+	1+		1+	3+	3+	3+	3+	HRSE
290	283	19	F	N	3+2+1+	3+		3+	-	3+	3+	3+	-	HR
291	286	33	F	FU	3+2+1+	3+	3+	-	3+	3+	-	3+	3+	HSE
292	290	20	F	Re	3+2+1+	-	-	3+	-	-	-	2+	-	-
293	293	42	F	Re	3+2+1+	3+	3+	-	-	-	-	3+	-	H
294	294	35	M	Re	3+2+1+	3+	3+	3+	3+	3+	3+	3+	3+	HRSE
295	297	27	M	FU	3+2+1+	-	-	-	-	-	-	-	-	-

Appendix VI: Statistical analysis

Statistical tool – Chi-square (X^2) test

1. Sexwise distribution of resistant *M. tuberculosis* cases.

	Sensitive	Resistant	Total
Male	71	146	217
Female	23	55	78
Total	94	201	295

Here, the null hypothesis (H_o) is that there is not significant relation between the sex and drug resistant isolates.

S.N.	Observed value (O)	Expected value (E)	$(O-E)^2$	$X^2_{cal} = (O-E)^2/E$
1	71	69.14	3.45	0.04989
2	23	24.85	3.42	0.13762
3	146	147.85	3.42	0.02313
4	55	53.14	3.45	0.06492 $\Sigma X^2_{cal} = 0.07556$

$\Sigma X^2_{cal} = 0.07556$

Degree of freedom (d.f) = (2-1) (2-1) =1

According to the table,

ΣX^2_{tab} at 5% level of significance for 1 d.f. =3.841.

Since, $\Sigma X^2_{cal} < \Sigma X^2_{tab,}$ so H_o is accepted i.e. there is not significant relation between the sex and drug resistant isolates.

2. The association of drug resistance pattern with family history of tuberculosis.

Type	Family history of TB patients		Total
	Present	Absent	
Drug resistance	21	50	71
Drug sensitive	32	47	79
Total	53	97	150

Here the null hypothesis (H_o) is that Drug Resistance are not related with family history of tuberculosis.

S.N.	Observed value (O)	Expected value (E)	$(O-E)^2$	$X^2_{cal} = (O-E)^2/E$
1	21	25.08	16.64	0.66
2	32	27.91	16.72	0.59
3	50	47.91	4.36	0.09
4	47	51.08	4.08	0.079 $\Sigma X^2_{cal} = 1.41$

$\Sigma X^2_{cal} = 1.41$

Degree of freedom (d.f) = (2-1) (2-1) =1

According to the table,

ΣX^2_{tab} at 5% level of significance for 1 d.f. =3.841.

Since, $\Sigma X^2_{cal} < \Sigma X^2_{tab}$ so H_o is accepted i.e. there is not any association between the drug resistance and family history of tuberculosis.

3. The association of drug sensitivity of *M. tuberculosis* in patients with smoking habit.

S.N	Drug susceptibility	Smoking		Total
		Present	Absent	
1	Drug resistance	28	43	71
2	Drug sensitive	37	42	79
Total		65	85	150

Here, the null hypothesis (H_o) is that there is not significance relation between the sex and drug resistant isolates.

S.N.	Observed value (O)	Expected value (E)	$(O-E)^2$	$X^2_{cal} = (O-E)^2/E$
1	28	30.76	2.76	0.0897
2	37	34.23	7.67	0.2241
3	43	40.23	7.67	0.1907
4	42	44.76	7.61	0.1701 $\Sigma X^2_{cal} = 0.6746$

$\Sigma X^2_{cal} = 0.6746$

Degree of freedom (d.f) = (2-1) (2-1) =1

According to the table,

ΣX^2_{tab} at 5% level of significance for 1 d.f. =3.841.

Since, $\Sigma X^2_{cal} < \Sigma X^2_{tab}$ so H_o is accepted i.e. there is not significant relation between drug resistant isolates and smoking habit.

4. Association of drug resistant *M. tuberculosis* with alcoholic habit of the patients.

Type	Alcoholic	Non-alcoholic	Total
Drug resistant	25	46	71
Drug sensitive	23	56	79
Total	48	102	150

Here, the null hypothesis (H_o) is that there is not any significant relation between the drug resistance and alcoholic habitat of the tuberculosis patients.

S.N.	Observed value (O)	Expected value (E)	$(O-E)^2$	$X^2_{cal} = (O-E)^2/E$
1	25	22.72	5.19	0.2288
2	23	25.28	5.19	0.2056
3	46	48.28	5.19	0.1076
4	56	53.72	5.19	0.0967 $\Sigma X^2_{cal} = 0.6387$

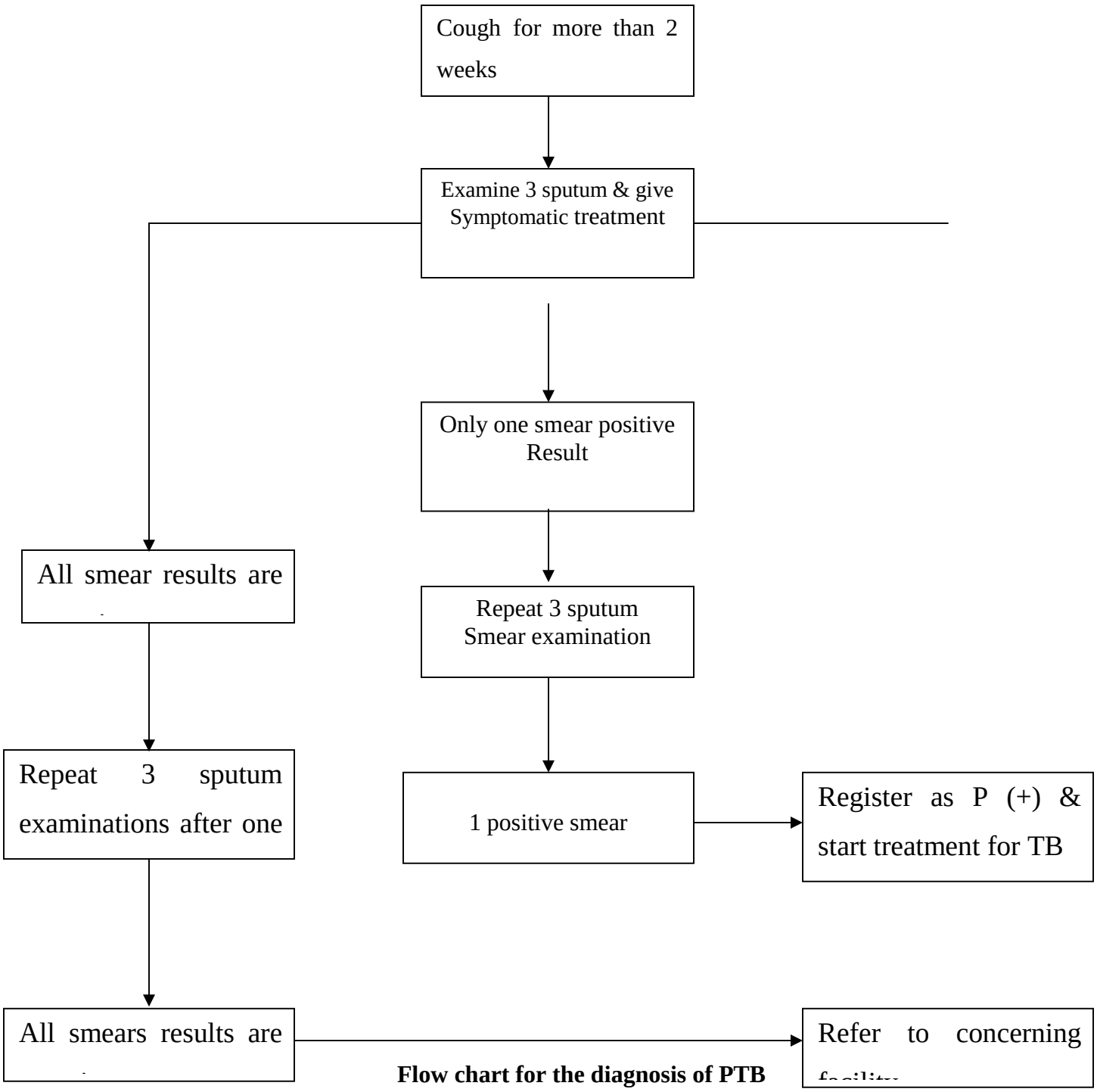
$\Sigma X^2_{cal} = 0.6387$

Degree of freedom (d.f) = (2-1) (2-1) =1

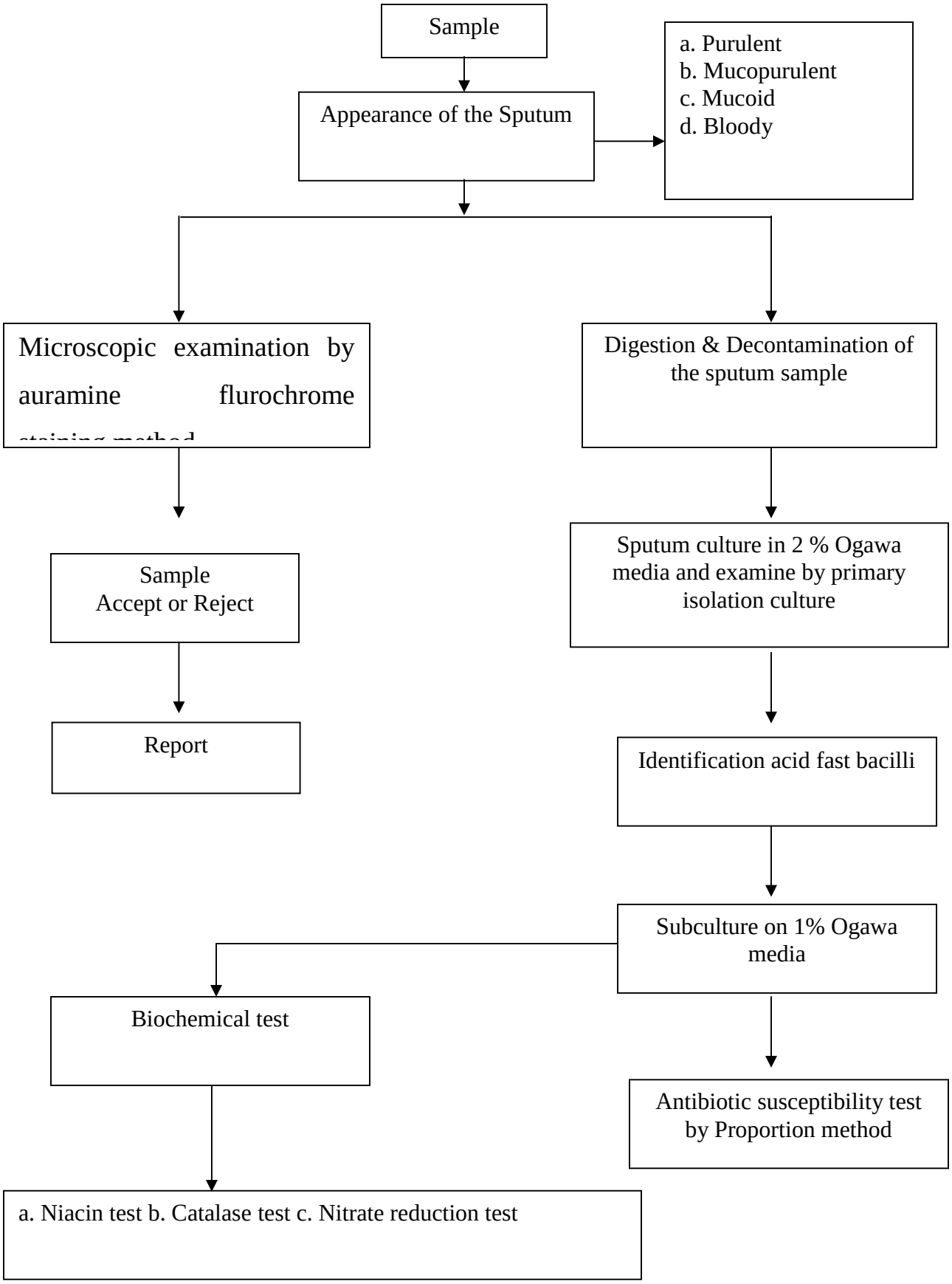
According to the table,

ΣX^2_{tab} at 5% level of significance for 1 d.f. =3.841.

Since, $\Sigma X^2_{cal} < \Sigma X^2_{tab}$ so H_o is accepted i.e. there is not any significant relation between the drug resistance and alcoholic habit of the tuberculosis patients.



Flow chart for the diagnosis of PTB



Flow chart Illustrating laboratory examination of sputum for *Mycobacterium tuberculosis*