CHAPTER I

1. INTRODUCTION

Globally, two billion people, equal to one-third of the world's population, are infected with tubercle bacilli. 1.6 million people died from tuberculosis (TB) in 2005 which is equal to an estimated 4,400 deaths a day. It is a leading cause of death among HIV/AIDS infected people. About 200,000 people living with HIV/AIDS die from TB every year, most of them being in Africa. Similarly, 450,000 new multi drug-resistant TB (MDR-TB) cases are estimated to occur every year (WHO, 2007).

In Nepal, about 45% of the total population is infected with TB, of which 60% are adult. Every year, 40,000 people develop active TB with 20,000 infectious pulmonary diseases. 5,000 to 7,000 people die of TB every year in Nepal. The latest survey conducted in 2001/2002 showed MDR-TB of 1.30%. In 2002, 2.4% of TB patients were infected with HIV (NTC, 2007).

Tuberculosis is among the most serious infectious cause of global morbidity and mortality. It is a disease of great antiquity having been mentioned in the "Vedas" as "rajayakshma". Evidence of TB lesions of bone has been found in Egyptian mummies dating back to 3400 B.C. In the past, TB was referred to as the "white plague" and by John Bunyan, as the "captain of all these men of death". WHO (2004) defines TB as a contagious disease which like common cold spreads through the air. TB is the chronic bacterial infection predominantly caused by *M. tuberculosis* which can be characterized by the formation of granuloma as a result of cell mediated hypersensitivity. It is one of the most prevalent communicable diseases throughout Nepal (NTC, 2007).

TB is a specific infectious disease primarily affecting lungs causing pulmonary tuberculosis (PTB) (Park, 2005). PTB usually occurs in the apex of the lung (WHO, 1998). It can also affect intestine, meninges, bones and joints, lymph glands, skin

and other tissues of the body. The disease is usually chronic with varying clinical manifestations. PTB is also called “open case” of TB because acid fast bacilli (AFB) can be detected in the sputum sample of the infected patients. According to WHO (2007), an untreated TB patient can infect on average 10-15 people every year and 1 in 10 people infected with TB bacilli will be sick with active TB in their lifetime. Sputum smear positive PTB case may be defined as the case in which the patient gives at least two sputum smear positive for AFB by microscopy, or at least one sputum specimen positive for AFB, which is culture positive for *M. tuberculosis.*

The cornerstone of the diagnosis of TB is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Sputum examination by microscopy is relatively quick, easy and inexpensive and must be performed on all cases suspected of having TB. Examination by bacteriological culture provides the definitive diagnosis of TB and is regarded as “gold standard” method. Culture increases the number of TB cases found, often by 30-50%, detects cases earlier, often before they become infectious (WHO, 1998). Cultures also provide the necessary material for drug susceptibility testing.

Identification of mycobacteria should never be done with a single test. Although, colony morphology and pigment production can aid in identification, final identification should never be based on these characteristics only. Mycobacterial growth in media to which inhibitory substances are added has been used in species identification. Growth of Mycobacterium tuberculosis complex (MTC) is inhibited by para-nitrobenzoic acid (PNB), whereas non-tuberculous mycobacteria are resistant. Though, ,various other biochemical tests are available, according to WHO (1998), one or more of the catalase tests together with growth at 25°C and growth on L-J medium containing PNB at 37°C can be combined together for the identification of tubercle bacilli.

Non-tuberculous mycobacteria (NTM) are the large number of mycobacterial species frequently found in environmental habitats that may colonize and

occasionally cause infection in humans and animals. Such infections are termed mycobacterioses. Mycobacterioses are becoming more prevalent with the increasing prevalence of immunocompromised hosts, particularly in relation to the AIDS pandemic (Hornick *et al.,* 1998). So, it is important to differentiate NTM from *M. tuberculosis*. They can be differentiated from *M. tuberculosis* by using PNB in culture medium. NTM can grow in culture medium containing PNB whereas *M. tuberculosis* cannot.

Cytochemical staining by using Neutral red is one of the most reliable methods for the identification of virulent strains of *M. tuberculosis*. To date, reliable differentiation of avirulent strains from virulent strains have been possible only through spoligotyping and IS *6110* genotyping, laborious, time consuming and not easily available procedures. Virulent strains of *M. tuberculosis* (along with $H_{37}Rv$) fix neutral red in an alkaline aqueous environment and become red in color, whereas avirulent strains do not.

According to WHO, more people will die of TB this year than in any other year in history. Of equal concern, however, are the emergence and transmission of Multi-Drug Resistant strains of *M. tuberculosis*. In light of this frightening scenario, laboratory strategies for reliable Antimicrobial susceptibility testing of *M. tuberculosis* are of prime importance. Antimicrobial susceptibility testing of *M. tuberculosis* is carried out with a primary set of drugs, consisting of the front line drugs- Isoniazid, Rifampicin, Ethambutol and Streptomycin, by proportion method.

Since, isolation, identification and drug susceptibility test are vital in TB bacteriology; this study is therefore, undertaken to screen *M. tuberculosis* by using PNB, virulence testing and drug susceptibility testing to primary anti-tubercular drugs.

CHAPTER II

2. OBJECTIVES OF THE STUDY

2.1 General objective

To screen *Mycobacterium tuberculosis* by selective inhibition with para-nitrobenzoic acid, its cytochemical staining and drug susceptibility to primary anti-tubercular drugs.

2.2 Specific objectives

1. To develop a test using PNB added to culture medium, and to evaluate its usefulness in the screening of mycobacterial isolates.
2. To confirm the screened isolates as *M. tuberculosis* by different biochemical tests.
3. To test virulence using neutral red cytochemical staining test.
4. To evaluate the drug susceptibility pattern of the confirmed isolates to primary anti-tubercular drugs.

CHAPTER III

3. LITERATURE REVIEW

3.1 Definition

Tuberculosis is a contagious disease of global importance (WHO, 1998). TB is a disease of great antiquity and has almost certainly caused more suffering and death than any other infection (Grange, 1998). Hence, TB can be defined as a chronic bacterial infection caused by *M. tuberculosis* and characterized by the formation of granuloma in infected tissues as a result of cell mediated hypersensitivity. TB of lungs (PTB) is much more common but may also have extra pulmonary extension affecting the lymph nodes, intestine, meninges, bones and joints, skin and other parts of the body with varying clinical manifestations such as, evening rise of fever, decreased appetite and weight loss, haemoptysis and progressive weakening of the body.

According to Pace (2000) TB is a disease caused by the body's attempt to control the multiplying and spreading of the bacterium, *M. tuberculosis.* TB is often the first disease to occur in the AIDS patient, even before any of opportunistic disease appears, and it is generally move intractable than in non-AIDS patient.

3.2 Historical overview

Tuberculosis has been a major cause of suffering and death since times immemorial. Thought to be one of the oldest human diseases, the history of tuberculosis is at least as old as the mankind (Sharma *et al.,* 2001). In all probability, TB is older than the human race (Madkour *et al.,* 2004).

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" (Rubin, 1995). There have been references to this ancient scourge as in the Vedas [vide infra] and it was called "rajayakshma" (meaning "wasting diseases"). In the Krishna Yajurveda Samhita, there

is reference to how Soma [Moon] had been affected by “yakshma”. Since “Soma” who was “the king and the ruler” was affected by “yakshma”, it came to be known as “rajayakshma”. Hippocrates [460-377 B.C.] called the disease “pthisis”, a Greek word which meant “to consume”, “to spit” and “to waste away” (Flick, 1925; Webb, 1936). Evidence of TB lesions of bone has been found in Egyptian mummies dating back to 3400 B.C. The word “consumption” [derived from the Latin word “consumere”] has also been used to describe tuberculosis in English literature. The Hebrew word “schachepheth” (meaning “waste away”) has been used in the Bible. J.L. Schonlein, professor medicine at Zurich, is credited to have named the disease “tuberculosis” (Rosenblatt, 1973). The word “tuberculosis” is a derivative of Latin word “tubercula” which means a “small lump” (Waksman, 1964; Dubos *et al.*, 1952). Several names have been used to refer to tuberculosis in the years gone by. Acute progressive TB has been referred to as “galloping consumption”. PTB has been referred to as “tabes pulmonali”. Similarly, tuberculosis cervical lymphadenitis has been called as “scrofula”, “king’s evil”, “stroma” and abdominal TB as “tabes mesenterica”. Cutaneous TB has been called “lupus vulgaris” and vertebral TB has been cited as “pott’s disease”. Oliver Wendell Holmes referred to disease as “white plague” (Waksman, 1964).

The transmissible nature of tuberculosis was clearly established by Jean-Antonie Villemin, a French military Doctor in 1868. Robert Koch discovered *M. tuberculosis* on 24 March 1882 and succeeded in culturing it on inspissated serum. In addition to culturing the causative organism, Koch succeeded in staining it by treatment with an alkaline solution of Methylene blue for 24 hours. The acid fast nature of the organism was discovered by Ehrlich in 1885 and the present method of acid-fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence the name Ziehl-Neelsen staining technique (ZN staining) was established.

Though the disease has been identified and the etiological agent was isolated earlier, the modern era of tuberculosis treatment began only in 1946 with the advent of Streptomycin and the development of Isoniazid in 1952.

3.3 Epidemiology

3.3.1 TB worldwide

Among communicable diseases, TB is the second leading cause of death worldwide after HIV/AIDS, killing nearly 2 million people every year (WHO, 2004). 2 billion people, equal to one-third of the world's total population, are infected with TB bacilli, the microbe that causes TB. It has been estimated that 1.6 million people died from TB in 2005, equal to approximately 4,400 deaths a day, including 195,000 patients infected with HIV (WHO, 2007). Almost 9 million new TB cases occurred in 2004 (WHO, 2006) and there were 8.8 million new TB cases in 2005 (WHO, 2007) with 80% of them in 22 countries.

According to WHO (2007), more than 90 million TB patients were reported to WHO between 1980 and 2005. 26.5 million patients were notified by DOTS programmes between 1995 and 2005, and 10.8 million new smear positive cases were registered for treatment by DOTS programmes between 1994 and 2004. TB annual incidence rates are now stable or falling in all six WHO regions and have peaked globally; however, the total number of cases is still rising in the African, Eastern Mediterranean and South East-Asian regions. HIV infection accounts for much of the recent increase in the global TB incidence.

3.3.2 TB in the South-East Asia Region (SEAR)

TB is a disease of poverty; affecting mostly young adults in their most productive years; the vast majority of TB deaths are in the developing world, with more than half of all deaths occurring in Asia (WHO, 2007). WHO estimates that the largest number of TB cases in 2004 occurred in WHO's SEAR, which accounted for 33% of incident cases globally. It is estimated that 1.7 million deaths resulted from TB in 2004. SEAR has the highest burden of tuberculosis cases among all the WHO regions with five countries- Bangladesh, India, Indonesia, Myanmar and Thailand, together contributing to 95 % of

the regional burden. According to WHO (2004), India has the largest number of TB cases, with an estimated 1.8 million (20% of the global TB burden). The rapidly expanding HIV epidemic in this region is a growing concern. Between 2.5 to 3 million people in the region are currently estimated to be infected with both TB and HIV.

3.3.3 TB in Nepal

Tuberculosis is a major public health problem in Nepal. About 45% of the total population is infected with TB, of which 60% are adult of productive age (15-45 years). Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. These 20,000 are able to spread the disease to others. Although introduction of treatment by DOTS has already reduce the number of deaths ; however 5,000-7,000 people still continue to die each year from TB (NTC, 2007).

As TB spreads easily in areas of high population density, many TB patients are found in the cities and terai areas. The annual rate of infection is estimated about 3% (Pokharel *et al.*, 2004). A concentrated HIV epidemic is one of the major problems in the treatment of TB in Nepal. In 2002, 2.4% of tuberculosis patients also had HIV infection. The estimated HIV prevalence among TB patients is 2.9% with estimated mortality of 261 (Mortality rate per 100,000 general population is 1.0) (WHO, 2004). In Nepal, DOTS strategy has been implemented for TB control since 1996 and has already reduced the number of deaths remarkably. Expansion of this cost effective and highly successful treatment strategy of DOTS, which already has proven its efficacy in Nepal, will have a profound impact on mortality and morbidity (NTC 2001/2002).

3.3.4 The molecular epidemiology of tuberculosis

The ability to track specific strains of *M. tuberculosis* as they spread through a population is critical for understanding of the transmission and pathogenesis of TB. Molecular genotyping techniques that allow to differentiate isolates of *M. tuberculosis* for the purpose of tracking strains in the community and designing prevention and

control strategies to block further transmission are now available. Molecular techniques can help address epidemiological problems that cannot be approached or would be more difficult to address by conventional methods. The first molecular based technique to subspeciate *M. tuberculosis* was a direct gel electrophoresis comparison of restriction digests of genomic DNA. Various other methods include IS *6110* RFLP (widely used method), PGRS typing, spoligotyping, and PCR and DNA sequence-based methods. These techniques have been used to estimate the fraction of cases attributable to recent transmission versus reactivation, confirm laboratory cross-contamination, differentiate recurrent TB into either endogenous reactivation or exogenous re-infection, identify host specific risk-factors and study different properties and pattern of drug resistance (Mathema *et al.,* 2004; Deriemer *et al.*, 2004).

3.4 Pathogenesis of tuberculosis

3.4.1 Infection and transmission

Tuberculosis is a contagious disease. Like common cold, it spreads through the air (WHO, 2004). TB transmission occurs almost exclusively from human to human; a pre-requisite is having contact with a source case. More than 80% of new TB cases result from exposure to sputum smears positive cases, although smear-negative, culture-positive cases can be responsible for up to 17% new cases.

Chakraborty (2003) describes three modes of infection:

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

It is well established that patients with sputum, that is positive on direct smear examination are the principal source of infection (Grange, 1994). Risk of infection is determined by closeness of contact, sources of infection and the immunostatus of the host. Risk especially increases in crowded and poorly ventilated areas.

Tuberculosis is spread by air borne droplet nuclei, which are 1-5 µm particles containing 1 to 400 bacilli each. They are expelled in the air with coughing, sneezing, singing laughing, talking etc. and remain suspended in the air for many hours. Coughing generates the largest number of droplet of all sizes (Park, 2005). One cough can produce 3000 to 5000 droplet nuclei. Transmission generally occurs indoors, where droplet nuclei can stay in the air for a long time. Ventilation removes droplet nuclei. Direct sunlight quickly kills tubercle bacilli, but they can survive in the dark for several hours. Two factors determine an individual's risk of exposure- the concentration of droplet nuclei in contaminated air and the length of time the patient breaths that air.

3.4.2 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. Certain groups have high risk for development of TB and they are refugees, physical and psychological stress, nursing home residents and the impoverished. Also the vulnerable groups or the individuals more susceptible to the disease include persons under conditions like, poorly controlled diabetes mellitus, chronic lung disease (bronchitis) and silicosis, cancer, advance kidney disease, malnutrition, alcoholism, disease for which steroid therapy is prescribed; heavy smokers, elderly, low income groups, intravenous drug users, living and attending drug treatment centers, hospital, nursing homes etc (WHO, 1993 and WHO, 1997).

3.4.3 Virulence factors of tubercle bacilli

The virulence of the tubercle bacilli is due to its resistance to tissue cells and fluids rather than to the production of toxic substances (Grange, 1994). The virulence of tubercle bacilli appears to be related to their ability to survive within Macrophages and the organisms exhibit long periods of metabolic inactivity. Mycobacterial virulence factors can have both direct and indirect effects on host cells and can ultimately lead to tissue destruction and disease. Direct cell-cell interactions, including attachment, invasion, and intracellular multiplication and indirect interactions, through secreted bacterial factors such as hemolysin and cytotoxin, can cause lysis of the host cells. In addition, lipoarabinomannans (LAMs), heat shock proteins, and Mycobacterial products can stimulate host cell to produce inflammatory products or cytokines that can amplify tissue damage in the host (Quinn *et al.,* 1997). Mechanisms hypothesized for how tubercle bacilli avoid being killed by macrophages include:

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

Several bacterial components have been implicated in these various hypotheses e.g. phenolic glycolipids can act as scavengers of free radicals and the Mycolic acid layer can a ct as a hydrophobic barrier to bactericidal agents. Similarly, a recently described cytolytic activity might be involved in the ability of mycobacteria to enter and exit host cells or perhaps escape from the phagosome. Phagocytized tubercle bacilli appear to inhibit acidification of the phagosome and the subsequent phagosome-lysosome fusion. Potential fusion-inhibiting substances made by the mycobacteria include ammonia,

polyglutamic acid and sulpholipids, although the key molecule has not been identified. Some electron microscopic evidence suggests that the tubercle bacilli escape from the phagosome and replicate in the cytoplasm whereas other data suggest that *M. tuberculosis* ends up in another membrane bound vacuole which contains proteins characteristic of phagosomes and, early and late endosomes (Good *et al.*, 1998)

Table 1. Known and suspected virulence factors of *M. tuberculosis* (Quinn *et al.*, 1997, with modification)

S. No.	Putative factors	Proposed activity
1	Invasion protein	Invasion of non professional phagocytes and intracellular survival
2	Fibronectin binding proteins	Adhesion and invasion
3	Complement and mannose receptor binding	Entry into professional phagocytes
4	Alternate phagosomal pathway (phagosomal trafficking)	Survival in the phagosome
5	Prevention of phago-lysosome fusion	Survival in the phagosome
6	Escape into the cytoplasm and budding into novel phagosome	Survival in the cytoplasm
7	Hemolysin/Phospholipase	Lysis of phagosome; leakage of the phagosome
8	Cytotoxicity	Tissue destruction and enhanced TNF effects
9	Lipoarabinomannons	Reduced TNF induction and macrophage effectors function
10	Heat shock proteins	Induction of inflammatory cytokines
11	Cord factor	Tissue destruction and necrosis
12	Sulfolipid	Induction of inflammatory mediators
13	Super oxide dismutase catalase	Inhibitors of reactive oxygen and nitrogen radicals within the phagosome
14	Exochelins mycobactin	Acquisition of ferric iron

3.4.4 Types of tuberculosis

3.4.4.1 Pulmonary tuberculosis (PTB)

Pulmonary TB refers to disease involving the lung parenchyma as the lung is the usual site of primary lesion and the principal organ involved. 80% of TB occurs in the lung and is called pulmonary tuberculosis (NTC, 1997).

The pathogenesis of PTB can be divided into primary and post primary infection.

3.4.4.1.1 Primary pulmonary tuberculosis

When tubercle bacilli enter the body on first exposure then primary infection occurs. Inhaled droplet nuclei (1-5 µm) avoid the mucocilliary defenses of the bronchi and lodge in the terminal alveoli of the lungs. When the tubercle bacilli multiplies, they form caseous and granulomatous focal lesion known as “Ghon focus”. Mycobacteria spread rapidly via the intrapulmonary lymphatic to the region hilar lymph nodes, where the organisms are ingested by the reticuloendothelial cells. Caseous and granulomatous lymphadenitis rapidly ensure, causing considerable hilar lymph node enlargement. The primary lung lesion (Ghon focus) and the regional lymph node lesions are together referred to as the primary complex.

Bacilli may spread in the blood from the primary complex throughout the body. The immune response (delayed hypersensitivity and cellular immunity) develops about 4-6 weeks after the primary infection. The size of the infecting dose of the bacilli and the strength of the immune response determine the prognosis of the disease. In most cases, some of them remain viable or ‘dormant’ for many years. This stage is called latent TB infection (LTBI), which is generally an asymptomatic, radiologically undetected process in humans. Sometimes a primary complex (Ghon complex) can be seen radiographically, mostly in the lower and middle lobes, and comprise the primary lesion, hilar lymphadenopathy plus/minus a lymphangitic track. Later on, the primary lesion tends to become calcified, and can be identified on the chest radiographs for

decades. Most commonly a positive tuberculin test remains the only proof of LTBI, and therefore does not signify active disease (WHO, 2004).

3.4.4.1.2 Post primary tuberculosis

Post primary TB occurs after a latent period of months or years after primary infections. It may occur by reactivation of dormant bacilli i.e. dormant bacilli persisting in tissues for months or years after primary infection start to multiply or by reinfection i.e. a repeat infection in a person who has already previously had a primary infection. Reactivation may be in response to a trigger, such as weakening of the immune system by HIV infection. Post primary TB usually affects the lungs but can involve any part of the body. The characteristics features of post primary PTB are: extensive lung destruction with cavitations, positive sputum smear, upper lobe involvement, usually no intrathoracic lymphadenopathy (Crofton *et al.*, 1999; Grange, 1998).

3.4.4.2 Extra pulmonary tuberculosis

Most, if not all, extra pulmonary lesions results by haematogenous spread of the organism from a primary focus which is not always detected (Chakraborty, 2003). The higher rate of infection of extra PTB cases in immunocompromised states is associated with old age, renal failure, malnutrition, hematological malignancies and HIV/AIDS. There are many types of extra pulmonary TB. These include pleurisy, gland, intestinal, miliary, meningitis, bone, urogenital, skin and eye TB (NTC, 1997). Patients usually present with constitutional features (fever, night sweats, weight loss) and local features related to the site of diseases are similar in adults and children. Many patients with extra PTB also have co-existent pulmonary TB.

3.5 Immunology of tuberculosis

Protective immune reactions in tuberculosis are principally cell-mediated. Humoral immune responses are usually, but perhaps rather dogmatically, regarded as having no protective role to play. The protective cellular immune responses macrophage

activation, granuloma formation, and recognition and destruction of exhausted macrophages and other cells in which tubercle bacilli are replicating. The outcome of infection by the tubercle bacillus depends critically on whether the host response with a protective or tissue-necrotizing reaction (Grange, 1998).

3.5.1 Early immunological events after infection

Tubercle bacilli entering the tissues are taken up by phagocytic cells, such as alveolar macrophages in the case of PTB. If the bacilli are not destroyed, they replicate and kill the cell. A local area of inflammation is thus established attracting more phagocytes. Some bacilli are transported, probably within phagocytes, to the regional lymph nodes where they are engulfed by antigen presenting cells (APC). Other bacilli are transported further afield and may cause one of the extrapulmonary forms of primary diseases such as tuberculous meningitis.

Epitopes from mycobacteria lying within phagosomes within the APC are presented on the cell surface by the MHC class II (HLA-D) molecules to CD4+ helper T-cells which undergo activation and clonal proliferation producing a range of cytokines e.g. IFN-γ that activates macrophage. If the tubercle bacilli proliferate within the APC and escape from the phagosomes, their epitopes are presented to CD8+ T-cells by MHC class I (HLA-A and B) molecules which may lyse these antigen presenting cells.

3.5.2 Macrophage activation and granuloma formation

In the early stage of active TB, most tubercle bacilli are within the macrophages that, paradoxically, are the cells best equipped to destroy them by various lysosomal enzymes and reactive oxygen intermediates (ROI). There are 3 strategies by which mycobacteria survive within macrophages. First, by inhibition of fusion of the phagosome to the lysosomes (poorly understood mechanism),second, they neutralize ROI by means of cell wall lipids including LAM and mycosides, and by secreting the

enzyme superoxide dismutase, third, they escape from the phagosome and replicate in the cytoplasm of the cell (Madkour *et al.,* 2004; Grange, 1998).

IFN-γ though activates macrophages; it is not enough to kill tubercle bacilli, so it also induces a hydroxylase within macrophage which converts vitamin-D into calcitriol activating cells further. In disease, activated macrophages aggregate to form characteristic lesion of TB and many other infections, namely, the granuloma. The outer regions of granuloma containing lymphocytes secrete IFN-γ thereby attracting more macrophages to the site. Thus, such lesions are termed "high turnover granulomas of immunogenic origin". The entire granuloma is more capable of destroying tubercle bacilli by maintaining anoxic condition at the centre. When the bacilli are killed, the granulomas become inactive; fibroblasts surround them with fibrin which then contracts to form scars which may become calcified.

3.5.3 Cytotoxic cells and protective immunity

Cytotoxic activity is much more important in the destruction of tubercle bacilli. Similarly, heat shock proteins (HSPs) of bacterial or host origin, or both, that are presented on cell surfaces during stress, may be target for cytolytic activity. In this respect, a population of CD4+ cytolytic cells recognizing a predominant mycobacterial HSP has been described and a major sub-population of γ/δ cells with cytolytic activity recognize HSPs on the surface of infected cells. There is also evidence that immune recognition of HSPs and other antigens common to the genus *Mycobacterium* is suppressed in patients with active TB. Skin testing with a new set of tuberculins prepared from different mycobacterial species reveals different categories of reactors.

3.5.4 The immune spectrum in tuberculosis

Many attempts have been made to categorize cases of tuberculosis according to an immunological "spectrum". According to Ridley and Ridley (1987) there are three

categories of TB, as shown in Table 2. Most patients in group 1 had chronic skin TB; those in group 2 had pulmonary disease whereas those in group 3 had disseminated TB.

Table 2. Grouping of tuberculosis according to histological features

Group	Main cell types	Necrosis	Giant cells	Bacilli
1a	Organized mature epitheloid cells	None	+	None
1b	Unorganized mature and immature epitheloid cells	Patchy fibrinoid	+	Rare
2a	Immature epitheloid cells	Caseation, no nuclear debris	+	Scanty
2b	Immature epitheloid cells and/or undifferentiated histiocytes	Necrosis with nuclear debris and polymorphs	+	+
3a	Scanty macrophages	Extensive, basophilic; coarse nuclear debris	--	++
3b	Very few macrophages	Extensive, eosinophilic; scanty nuclear debris	--	+++

All forms of immunosuppression predispose to TB due to primary infection, endogenous reactivation or exogenous reinfection and the resulting disease differs from that in the more immunocompetent. Not only is there a failure to develop the characteristic high turnover granuloma of immunogenic origin in immunosuppressed TB patients, there is also a suppression tissue-necrotizing reactions and scar formation that would otherwise limit the spread of infection. Thus, discrete pulmonary lesions and cavity formation are both less common in such patients. Instead, there may be a radiologically rather non-specific spreading pulmonary lesions.

3.5.5 Genetic factors in mycobacterial immunity

Because not all those exposed to the tubercle bacillus develop overt TB, some form of genetically determined innate resistance to disease has long been suspected. Linkage of susceptibility to TB and the class I determinants (HLA-A and HLA-B) of the MHC is weak and varies from region to region but significant associations with class II (HLA-D) determinants have been found. Thus the HLA-DR2 gene predisposes to the development of advanced, smear-positive, PTB. This determinant may affect antigen recognition as it is associated with high levels of antibody to 38 kDa protein unique to the MTC. Though Nramp (natural-resistance-associated macrophage protein) like protein is found in human which codes for RNI, human macrophages appear unable to generate sufficient RNI to kill tubercle bacilli and it may be of less significance.

3.6 Blood examination during tuberculosis

Following changes in blood has been described by Crofton *et al.* (1999):

- Marked anemia is rarely caused by PTB but is sometimes seen in obscure ("cryptic") miliary TB. Anemia is more likely to be due to other causes e.g. worms or malnutrition.
- The white blood cell count is usually normal or low normal (it is often raised in pneumonia).
- A raised ESR may occur, but a normal result does not exclude active TB. It is therefore not a useful test and not worth doing.
- Low serum sodium and potassium may occur in severe disease and can cause death.
- Both lymphocytosis and lymphocytopenia are reported. An active TB causes a decrease in total T-cells secondary to decrease in T4 cells. Total B-cells are also decreased. Multiple cytokines, including IL and TNF are activated.

3.7 HIV/AIDS and Tuberculosis

HIV and tuberculosis have been described as the “Diabolical Duet” because when HIV infection increases, so does TB (NTC, 2007). HIV and TB form a lethal combination, each spreading the other’s progress. HIV weakens the immune system. Someone who is HIV-positive and infected with TB bacilli is many times more likely to become sick with TB than someone infected with TB bacilli who is HIV-negative. TB is a leading cause of death among people who are HIV-positive. In Africa, HIV is the single most important factor determining the increased incidence of TB in the past 10 years (WHO, 2004). According to WHO (2007), about 200,000 people are living with HIV/AIDS die from TB every year, most of them being in Africa. The prevalence of HIV is rising rapidly in Nepal, and effective control measure-for AIDS as well as for TB-are more important now than even before. In 1995/96, 0.60% of TB patients had HIV infection which had increased to 2.44% in 2002 (NTC, 2007). A healthy person who has been infected with TB has less than a 10% lifetime chance of developing active TB, whereas an HIV infected person who is also infected with TB has up to 10% chance each year of developing a life threatening case of TB (WHO, 2004).

3.8 Microbiology of tuberculosis

3.8.1 Etiology

The most predominant human pathogen is *Mycobacterium tuberculosis*, the etiological agent of human TB. Together with other highly related bacteria, *M. tuberculosis* forms a tightly knit complex, a single species as defined by DNA/DNA hybridization studies and characterized by a singular lack of genetic diversity. The complex includes *M. tuberculosis*, the causative agent in the vast majority of human tuberculosis; *M. canettii*, a smooth variant that is very rarely encountered; *M. africanum*, an agent of human TB in sub-Saharan Africa; *M. microti*, the agent of TB in voles; *M. bovis*, which infects a wide variety of mammalian species including

humans; and bacille Calmette-Guéruin (BCG), an attenuated derivative of *M. bovis* that is used as a live vaccine against TB (Room *et al.*, 2004).

The genus *Mycobacterium* is currently the only genus in the family Mycobacteriaceae, order Actinomycetales. There are currently 71 recognized or proposed species of *Mycobacterium*, that are medically classified into Mycobacterium tuberculosis complex (MTC) and Mycobacteria other than tuberculosis bacilli (MOTT). MOTT has been given several collective names-atypical, anonymous, non-tuberculosis, tuberculoid, opportunistic and paratubercle (Collee *et al.*, 1996).

3.8.2 Bacterial morphology

Mycobacteria are non-motile, non-sporing, weakly gram positive, aerobic or micro-aerophilic, straight or slightly curved rod-shaped bacteria. Some mycobacteria display coccobacillary, filamentous or branched forms and some produce yellow to orange pigment in the dark or after exposure to light. Their sizes vary from 1-4 µm × 0.3-0.6 µm in size, which frequently form small clumps.

M. tuberculosis (Human tubercle bacilli) are non-motile, non-sporing, non-capsulated and straight or slightly curved rods of about 2-3 × 0.2-0.4 µm in size (Chakraborty, 2003). Cell wall contains N-glcolylmuramic acid in lieu of N-acetylmuramic acid and has rich in chemically diverse lipids (up to 60% of the cell wall) causing them to grow as extremely rough hydrophobic colonies. The thickness of the cell wall is due to the presence of long-chain fatty acids (Mycolic acids) which form a thick palisade. Mycobacteria are described as being acid-alcohol fast. The basis of the acid-fast staining reaction is not clearly understood, but appears to be related to the presence of Mycolic acids in the cell wall, to the integrity of the cell and to the viability of the cell (Good *et al*., 1998).

3.8.3 Clinical and radiological diagnosis of pulmonary tuberculosis

The most important symptoms in the selection of TB suspects in adults (over 15 years of age) include: productive cough of more than 3 weeks, or haemoptysis, and significant weight loss. Patients with TB may also have other symptoms (which are more common, but less suggestive) such as: chest pain, breathlessness, fever/night sweats, tiredness, and loss of appetite) (WHO, 1997).

Chest radiology is often used for suspected TB patients, where and when available, as evidence of TB disease. Even very experienced doctors looking at chest X-ray find it extremely difficult to differentiate between active tuberculosis; other diseases and old healed TB. If X-ray is available, diagnosis of TB should not be made on a single abnormal X-ray (except for miliary TB) (NTC, 1997). Chest radiography can undoubtedly be very helpful in localizing abnormalities in the lung but to establish the TB etiology of an abnormality further examination is necessary and only bacteriology can provide the final proof (Toman, 1979).

3.8.4 Laboratory diagnosis of pulmonary tuberculosis

The cornerstone of the laboratory diagnosis of TB is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Examination by bacteriological culture provides the definitive diagnosis of TB (WHO, 1998).

3.8.4.1 Specimen collection and transport

For successful culture of specimens the time between specimen collection and the culturing process should be kept to a minimum. Although, *M. tuberculosis* is capable of causing disease in almost any organ of the body, more than 85% of TB disease in high prevalence countries is pulmonary. Therefore, sputum is the specimen of choice in the investigation of TB and should always be collected (WHO, 1998). Specimens should be collected in sterile, leak proof, disposable and appropriately labeled containers and placed into bags to contain leakage should it

occur (Forbes *et al.*, 2002). Pulmonary secretions may be obtained by any of the following methods: spontaneously produced or induced sputum, gastric lavage, transtracheal aspirations, brochoscopy, and laryngeal swabbing. Sputum samples should not be saliva but should be coughed up from the lungs. Because of the high yield among multi-bacillary cases, the IUATLD (1998) has recommended the "on-the-spot-early morning-on-the-spot" collection of sputum. Three or more consecutive samples should be examined, collected first thing in the morning if possible (Collee *et al.*, 1996). If sputum specimens can be kept refrigerated they could be sent to the laboratory once a week and if they have to be transported at ambient temperature, chemical preservation may be used (WHO, 1998). Three methods provide reasonable results, *viz*:

- Mixing the fresh specimen with an equal volume of 1% cetyl pyridinium chloride in 2% sodium chloride. Tubercle bacilli will survive for up to a week, while the growth of unwanted organisms will be restricted.
- Mixing the fresh specimen with anhydrous sodium carbonate in the proportion of 50 mg reagent to 2 ml specimen.
- If the delay before the cultural examination is to be less than 24 hours the specimen may be mixed with an equal volume of 23% trisodium phosphate.

However, none of the above mentioned preservation methods is optimal and speedy transportation is essential for good results.

3.8.4.2 Macroscopic examination

The gross features of TB are usually sufficiently distinctive to allow a tentative diagnosis to be made and to take precautions for avoiding infections. Macroscopic examinations include the observation of the color and appearance of the sputum sample-mucoid, salivary, purulent, mucopurulent, bloody sputum specimen (Cheesbrough, 2002). Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume.

3.8.4.3 Microscopic examination

AFB-microscopy can be used for accurate diagnoses, which is rapid with high specificity (99%). The main disadvantage is low sensitivity (25-75%). Similarly, microscopy can not distinguish between live and dead AFB, so that some patients excreting non-viable bacilli at the end of the treatment may be roughly considered as failure-cases. Mycobacteria retain the primary stain even after exposure to decolorizing acid-alcohol, hence the term "acid-fast". A counter-stain is employed to highlight the stained organisms for easier recognition. There are several methods of determining the acid-fast nature of mycobacteria.

Two most widely used staining techniques to observe AFB are ZN staining and Fluorescence method. ZN is the only method providing consistently good results without need for special equipment, and the required binocular microscope can be used for other purposes as well. In the carbolfuchsin (ZN) procedure, AFB appears red against a blue background. Fluorescence microscopy is cost effective and rapid if more samples are to be proceed each day. Fluorescence staining utilizes basically the same approach as ZN staining, but carbol fuchsin is replaced by the fluorescent dye, the acid for discoloration is gentler, and the counterstain is not essential although useful to quench background fluorescence. In Fluorochrome method, the acid-fast organisms appear as fluorescent rods, yellow to orange (the color may vary with the filter system used) against a paler yellow or orange background (IUATLD, 1998; WHO, 1998).

Other methods for light microscopy include cold staining techniques (such as with Kinyoun's or Gabett's solution, or Tam Tham Hok's method). For fluorescence microscopy, other methods include staining with Auramine/rhodamine, rhodamine, acridine orange, and others. These methods are reliable but are more expensive so not used in the routine examination.

3.8.4.4 Culture of sputum sample

Culture is the "gold standard" for diagnosis of TB which is most sensitive than AFB staining method and can reliably find mycobacteria when they are present in a concentration of about 10^3 organisms/ml of specimen. Various steps have to be followed before the inoculation of sample for culture. They are as given below:

3.8.4.4.1 Homogenization and decontamination

Decontamination of sputum specimens has two objectives:

- Destruction of bacteria other than mycobacteria ("decontamination"), and
- Homogenization of the specimen ("sputum")

Theoretically, there are many techniques available; none of them is ideal, i.e., none of them will selectively destroy only contaminating flora and achieve the complete liquefaction of the specimen. Indeed, what is needed is a reasonable compromise, i.e., to destroy as much of the contaminating flora as possible while harming as few mycobacteria as possible. Furthermore, all reagents needed should be inexpensive and readily available (IUATLD, 1998).

Sodium hydroxide (Modified Petroff) method is widely used in developing countries like ours, because of its relative simplicity and the fact that the reagents are easy to obtain. Other methods that can be employed for homogenization and decontamination includes N-acetyl-cysteine-sodium hydroxide (NALC-NaOH) method, Zephiran-trisodium phosphate (Z-TSP) method, Oxalic acid method, Sulphuric acid method and Cetylpyridinium chloride/bromide method for transit purpose. Specimens other than sputum demand even more care during processing because of the low number of tubercle bacilli present in positive specimens.

3.8.4.4.2 Culture media for isolation of mycobacteria

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

Solid media

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

Liquid media

Liquid media are not generally employed for routine cultivation but are used for sensitivity test, biochemical tests and preparation of antigens and vaccines. Mycobacteria grow as a surface pellicle in liquid medium unless a surfactant, such as the non-ionic detergent Tween 80, is added to lower the surface tension and permit diffuse growth. In general, the use of liquid media system reduces the turnaround time

for isolation of acid-fast bacilli to approximately 10 days, compared with 17 days or longer for conventional solid media (Forbes *et al.,* 2002). Growth of mycobacteria in liquid media, regardless of the type, requires 5-10% CO_2 gas. Virulent strains often grow as twisted rope like colonies called as" serpentine cords". Different types of liquid media are available for the isolation of mycobacteria like Herman Kirchner liquid medium, Dubos oleic acid-albumin liquid medium etc.

Biphasic media

Another media use for the culture of mycobacteria in some laboratories is the BBL-Septi-Chek AFB system, which is biphasic medium, containing both agar and broth. It uses Middlebrook 7H9 broth in its lower chamber and agar slope of Middlebrook 7H11 in the upper part. Mycobacteria readily grow in this medium and at 2 or 3 days intervals during incubation the broth is made run over the agar. Colonies are farmed on the agar if they contain mycobacteria.

Liquid automated culture systems

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

3.8.4.4.3 Inoculation and incubation

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

centrifuged sediment, distributed over the surface. Two slopes are inoculated per specimen.

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

3.8.4.5 Identification of *Mycobacteria species*

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

3.8.4.5.1 Growth rate and growth at 25°C, and 42°C

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

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

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

37°C and does not grow at all at 25°C or on PNB medium. According to Fujiki (2001), *M. tuberculosis* does not grow on PNB at 4 weeks of incubation and even if few colonies are observed on the medium, it should be regarded as negative. All other mycobacteria are resistant to PNB.

3.8.4.5.3 Niacin test

Although, all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its diagnosis. Before the niacin test is done, cultures should be checked for purity by microscopy; be 3-4 weeks old on egg based medium; and have sufficient growth of 50 or more colonies. The test may be done either with chemical reagents or with commercially available paper strips. Regardless of the method used, niacin is usually detected by its reaction with a cyanogens halide in the presence of the primary amine (Kent *et al.*, 1985).

3.8.4.5.4 Catalase test

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

3.8.4.5.5 Nitrate test

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allow this test to be used in combination with the niacin test in differentiating *M.*

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

3.8.4.5.6 Urease test

The ability of a culture to hydrolyze urea releasing ammonia ($NH_2CONH_2 + H_2O$ gives $2NH_3 + CO_2$) is useful in identifying both scotochromogens and non-photochromogens. *M. scrofulaceum, M. flavescens, M. bovis M. tuberculosis*, and *M. gastri* are positive, whereas *M. avium* complex, *M. xenopi*, *M. terrae* complex, and *M. gordonaei* are negative.

3.8.4.5.7 Pyrazinamidase test

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

3.8.4.5.8 Thiophen-2-Carboxylic acid Hydrazide (TCH) susceptibility test

This test is valuable for distinguishing *M. bovis* from *M. tuberculosis* and other nonchromogenic slowly growing mycobacteria. Only *M. bovis* is susceptible to low concentrations of TCH, 1-5 µg/ml. Isoniazid-resistant strains of *M. bovis* may be resistant to TCH. *M. tuberculosis* and other mycobacteria are usually resistant to the inhibitory action of this compound.

Various other tests are also available that can differentiate between *M. tuberculosis* and other mycobacteria e.g. Arylsulfatase test, Iron uptake test, Growth on MacConkey agar without crystal violet, Tellurite reduction, Tween hydrolysis, Tween opacity test etc.

3.8.4.5.9 Cytochemical staining with neutral red

At present, *M. tuberculosis* identification is normally carried out in clinical laboratories by using conventional tests such as niacin, nitrate reduction, pyrazinamidase, TCH, or catalase. In addition, MTC can be distinguished from other mycobacteria by commercialized nucleic acid and high-performance liquid chromatography (HPLC) of mycolic acids. However, it is not possible to distinguish the attenuated strain (avirulent strain) from *M. tuberculosis* clinical isolates by use of these techniques. Only spoligotyping and IS *6110* genotyping are available till date for the virulence test (Soto *et al.,* 2002; Hughes *et al.,* 1954).

In 1948, Dubos and Middlebrook described the neutral red cytochemical reaction that clearly distinguishes H_{37}Rv from avirulent or attenuated strains (Morse *et al.,* 1953). In alkaline aqueous solution of neutral red, the cells of the virulent H_{37}Rv *M. tuberculosis* strain fixed the dye and become red in color (Neutral red positive), whereas the cells off the avirulent strain (H_{37}Ra) remains unstained in the same condition (Neutral red negative). Though, sulfolipid is believed to be responsible for the virulence of the virulent strains, there have been isolation of virulent strains which lack sulfolipid compound (Soto *et al.,* 2002).

3.8.5 Molecular techniques for identification of mycobacteria

Since conventional methods for the identification of mycobacteria take 2-3 or more weeks, there has been increased effort to develop rapid identification techniques, especially in the wake of the resurgence of TB as a result of the HIV/AIDS pandemic. Molecular techniques have been developed in the last decade with the aim of achieving faster commencement of appropriate treatment, shortening time to detection of anti-mycobacterial drug resistance and instituting appropriate control measures. There are various types of molecular techniques so far developed, some of which are Nucleic acid probe method, Accuprobe system, BD probe Tec ET, Amplified *M. tuberculosis* (MTD) test, Amplicor *M. tuberculosis* (PCR) test, Ligase chain reaction (LCR), Thin layer

chromatography, HPLC, Gas liquid chromatography (GLC), Bactec 460 TB rapid radiometric culture system, MGIT, FAST plaque TB test and other different tests (Forbes *et al.,* 2002; Madkour *et al.,* 2004).

3.8.6 Serological diagnosis of tuberculosis

Since 40-60% of patients with pulmonary disease and up to 75% of patients with extrapulmonary diseases are smear negative, a number of alternative diagnostic methods using molecular, chromatographic and immunological methods have been developed (Madkour *et al.,* 2004). A number of serological tests developed for the diagnosis of TB have now become commercially available and these tests can be divided into 2 groups, they are:

3.8.6.1 Immunochromatographic tests

"ICT TB test" is an immunochromatographic test in which 5 highly purified antigens secreted by *M. tuberculosis* during active infection are immobilized in 4 lines on the test strip. The test detects the presence of IgG antibodies to these antigens. Similarly, "Rapid test TB" is a one step colored immunochromatographic assay which detects antibodies to the recombinant 38 kDa antigen from *M. tuberculosis* expressed and purified in *E. coli.*

3.8.6.2 Enzyme linked immunosorbent assay

"TB IgA EIA" test detects the IgA antibodies to a mycobacterial kp90 immuno-cross-reactive antigenic compound (ImCRAC). Similarly, "The pathozyme TB complex test" detects serum IgG antibodies to a recombinant 38 kDa antigen from *M. tuberculosis* expressed and purified from *E. coli*, permitting the isolation of significant quantities of protein. This antigen has been reported as the single most important antigen for the serological diagnosis of TB as it is a unique disease-associated protein that appears to be completely specific to the MTC. "PATHOZYME-MYCO IgG, IgA and IgM test" is also an ELISA based test that measures the levels in serum of IgG, IgA and IgM,

respectively to 2 antigens LAM and recombinant and recombinant 38 kDa antigens. These kits detect infections due to mycobacteria species.

3.8.7 Tuberculin testing

The tuberculin test is the only means of estimating the prevalence of infection in a population (Park, 2005). There are three types of tuberculin tests: the mantoux intradermal test, Heaf test and the Tine multiple puncture tests (Crofton *et al.,* 1999). The tuberculin test is useful in children aged 5 years and under, when a positive test suggests recent infection with TB and a high risk of developing disease (NTC, 1997).

Tuberculin is a purified protein derivative from tubercle bacilli which when injected into the skin of an infected person, produces a delayed local reaction after 24-48 hours. This reaction is quantified by measuring the diameter of skin indurations at the site of the reaction. . A “positive” tuberculin test does not mean the patient has active TB, and a “negative” tuberculin test does not exclude TB. A positive test may be due to infection with mycobacteria other than *M. tuberculosis* or *M. bovis.* The conditions which may suppress the tuberculin skin test include HIV infection, malnutrition, severe bacterial infection (including TB itself, Miliary TB), viral infection, cancer and use of immunosuppressive drugs (WHO, 1997).

3.8.8 Antimicrobial susceptibility testing (AST)

There are various methods of AST. They can be summarized under following headings:

3.8.8.1 Conventional methods of susceptibility testing

There are three general methods used throughout the world for determining drug susceptibility of mycobacteria: the absolute concentration method, the resistance ratio method, and the proportion method.

The absolute concentration method uses a standard inoculum grown on control media and media containing appropriately graded concentrations of the drug(s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits all or almost all of the growth; i.e. minimal inhibitory concentration (MIC). This method is widely used in Middle and Eastern Europe.

The resistance ratio method compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain. Parallel sets of media, containing twofold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown and known (standard) strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test (unknown) strain divided by the MIC for the standard strain in the same set. This method was developed by the British primarily for testing susceptibility of *M. tuberculosis* to streptomycin.

The proportion method enables precise quantitation of the proportion of mutants resistant to a given drug. Two serial dilutions are made from the original suspension of culture- 10^{-2} and 10^{-4}, using the calibrated inoculating loop (3 mm diameter). One set of control and two slopes of each drug containing media are inoculated with a loopful (0.01 ml) of each dilution (IUATLD, 1998). Seeded media are examined for growth and compared with the sets of control at critical concentrations of each drug, to label them as sensitive or resistant.

3.8.8.2 Rapid methods of susceptibility testing

Rapid methods of AST may be either radiometric methods or non-radiometric methods. Bactec 460 TB system is a radiometric method which gives the susceptibility testing result within 2 weeks. MGIT and MB/Bac T microbial detection system are non-radiometric methods of AST. Genetic approach to the drug resistance by *M tuberculosis* includes “Line probe assay method” which is based on the detection of mutations by hybridization in the rpo B gene.

3.9 Anti-tuberculosis chemotherapy

Chemotherapy is strictly antimicrobial treatment. Therefore its effect should be judged not by the anatomic healing of lesions but by their sterilization, or at least by the elimination of bacilli from the sputum (Toman, 1979).

3.9.1 Susceptibility of Mycobacteria to antimicrobial agents

Diseases caused by *Mycobacteria spp.* are difficult to treat. Most common drugs used in the treatment of TB patients include-Isoniazid, Rifampicin, Streptomycin, Ethambutol and Pyrazinamide.

Isoniazid (INH) has been the mainstay of anti-tuberculosis therapy for more than 40 years, but neither the target site in the mycobacterial cell nor the mode of action is well understood. The MIC of INH for susceptible strains of *M. tuberculosis* is usually less than 0.05 µg/ml. Rifampicin is a broad spectrum antibiotic which is active against *M. leprae, M. kansasii, M. haemophilum and M. marinum* as well as bacteria in other genera. RFP is involved in inhibition of DNA-dependent RNA polymerase. Streptomycin is an aminoglycoside that has been used extensively in the treatment of TB and it is active against in vitro against *M. tuberculosis*, *M. kansasii*, and *M. marinum.* Ethambutol is a synthetic compound, unrelated to previous anti-tuberculosis drugs. It is effective against drug-resistant strains of *M. tuberculosis* and some other mycobacteria. EMB inhibits cell wall synthesis and bacteriostatic. Pyrazinamide is a synthetic derivative of nicotinamide which is rapidly bactericidal for reproducing cells of *M. tuberculosis* and the average MIC is 20 µg/ml. Neither mechanism of action nor the molecular mechanism for resistance to PZA is fully known (Good *et al.*, 1998; Toman, 1979). Other drugs are given to TB patient according to the level of treatment and requirement e.g. Ethionamide, Para-aminosalicylic acid, Thiacetazone, Cycloserine, Kanamycin, Viomycin, Capreomycin etc.

3.9.2 Treatment regimens

The chemotherapeutic regimens are based on standardized combinations of 5 essential anti-TB drugs viz: INH, RFP, SM, EMB and PZA/Z.

Each standardized chemotherapeutic regimens consists of 2 phases:

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

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

Category I

These patients are:

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

Recommended regimens

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

Continuation phase: 4HR or $4H_3R_3$ i.e. Isoniazid and Rifampicin given daily or three times a week for 4 months. For patients with TB meningitides, disseminated TB or

Spinal disease with neurological complications, Isoniazid and Rifampicin should be given daily for 6-7 months (i.e. a total of 8 months of therapy).

Category II

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

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

Recommended regimens

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

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

Category III

These patients are:

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

Recommended regimens

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

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

Table 3. Recommended treatment regimens for each treatment category (WHO, 1997)

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

3.9.3 Adverse effects of anti-TB drugs

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 the reduced dose. The patient also receives symptomatic treatment. If a patient develops a major side effect, the treatment or the offending drug is stopped. Patients with major adverse reactions should be managed in a hospital (WHO, 1993; WHO, 1997; WHO, 2004).

Table 4. Symptoms based approach to adverse effects of anti-TB drugs

Side effects	Drug (s) probably responsible	Management
Minor		
Anorexia, nausea, abdominal pain	Rifampicin	Continue anti-TB drugs, check drug doses. Give drugs last thing at night.
Joint pains	Pyrazinamide	Aspirin
Burning sensation in feet	Isoniazid	Pyridoxine 100 mg daily
Orange/ red urine	Rifampicin	Reassurance

Major		
Itching of skin, skin rash	Thioacetazone (Streptomycin)	Stop anti-TB drugs
Deafness	Streptomycin	Stop Streptomycin, use Ethambutol
Dizziness	Streptomycin	Stop Streptomycin, use Ethambutol
Jaundice	Most anti-TB drugs (especially Isoniazid, Pyrazinamide and Rifampicin)	Stop anti-TB drugs
Vomiting and confusion (suspect drug-induced acute liver failure)	Most anti-TB drugs	Stop anti-TB drugs, urgent liver function tests and prothrombin time.
Visual impairment	Ethambutol	Stop Ethambutol
Shock, purpura, acute renal failure	Rifampicin	Stop Rifampicin

3.10 Drug resistant tuberculosis

Drug resistant TB is a case of TB (usually pulmonary) excreting bacilli resistant to one or more anti-tuberculosis drugs. Resistance of *M. tuberculosis* to anti-TB drugs is the result of a spontaneous genetic event and, worse "a man-made amplification of the natural phenomenon" (Pfyffer, 2000). Drug-resistant TB is caused by inconsistent and partial treatment, when patients do not take all their medicines regularly for the required period because they start to feel better, because doctors and health workers prescribe the wrong treatment regimens, or because the drug supply is unreliable (WHO, 2004).

3.10.1 Types of drug resistance

"Acquired or Secondary" resistance occurs in patients with some record of previous treatment (WHO, 2002). This is mostly because of single drug-due to irregular drug supply, inappropriate prescriptions or poor adherence to treatment-suppresses the growth of susceptible bacilli to that drug but permitted the multiplication of drug resistance organisms.

“Primary or Pretreatment” resistance 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.

Factors associated with anti-tuberculosis drug resistance:

According to Rijal *et al.* (2002), the factors associated with anti-tuberculosis drug resistance are:

- Programmatic factors:
  - Lack of standardized therapeutic regimen,
  - Poor implementation compounded by frequent or prolonged shortage of drug supply in areas with inadequate resources,
  - Political instability,
  - Use of anti-tuberculosis drugs of unproven quality.
- Health provider related factors:
  - Departure from the correct management of individual cases,
  - Selection of inappropriate regimen due to lack of recognition of prior treatment,
  - Ignorance of importance of standardized regimens,
  - Addition of a single drug to a failing regimen,
  - Lack of proper monitoring and supervising patients while on therapy.
- Patients related factors:
  - Non-adherence to prescribed treatment,
  - HIV infection.

3.10.2 Mechanism of resistance

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

Resistance to RFP arises due to mutations in the beta subunit of RNA polymerase encoded by rpo B, inhibiting RNA synthesis. Missense mutation in RNA polymerase and alteration in cell wall permeability is responsible for RFP resistance. Similarly, resistance to INH could occur either through a misssense mutation that blocks INH from binding to the gene or through formation of large amounts of enzyme which could nullify the effect of INH. Kat G gene product has also been shown to be responsible for INH resistance. Resistance to streptomycin emerges through mutations in rrs and rpsl gene that produce alteration in the streptomycin binding site. Most pyrazinamide resistant organisms have mutations in the pyrazinamidase gene, although the gene may also be inactivated through the insertion of IS *6110* (Gillespie, 2002).

3.10.3 MDR-TB: Multi-drug resistant TB

MDR TB refers to *M. tuberculosis* isolates that are resistant to at least both INH and RFP, the two most powerful anti-TB drugs. This is a very serious problem. People with MDR TB disease can only be treated with reserve or second-line drugs. These drugs are not as effective as the first-line drugs and cause more side effects.

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

3.10.4 XDR-TB: Extensive drug-resistant TB

XDR-TB, is currently defined as TB caused by organisms resistant to at least Isoniazid and Rifampicin among the first line drugs (i.e. MDR-TB) and to at least three of six main classes of second line anti-tuberculosis drugs (SLDs i.e. Aminoglycosides, Polypeptides, Fluoroquinolones, Thioamides, Cycloserine and Para-amino salicylic acid) (Raviglione, 2006). Researches have reported two cases of TB in Italy that were resistant to every single drug currently available to treat TB (WHO, 2007). Very little data is available from countries in SEAR. Detection of XDR-TB requires drug susceptibility testing for resistance to SLDs, which is technically difficult and only done at a very small number of laboratories. Further research regarding XDR TB is still on.

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

All the materials used in the study are listed in Appendix II.

4.2 Methods

4.2.1 Study setting

This study was the collaborative study between the Central Department of Microbiology, Tribhuvan University, Kathmandu and National Tuberculosis Centre, Thimi, Bhaktapur.

4.2.2 Study design

This cross-sectional study was carried out to screen *Mycobacterium tuberculosis* by selective inhibition with para-nitrobenzoic acid, its cytochemical staining with neutral red and drug susceptibility test to primary anti-tubercular drugs.

4.2.3 Study population

A total of 857 patients visiting National Tuberculosis Centre, Thimi, Bhaktapur were included in this study. The suspected patients were examined for AFB by fluorescence microscopy using Auramine dye. Only smear positive samples were further processed for the study.

4.2.4 Study period

The study was carried out during September 2006 to June 2007. All patients included in the study were attending NTC, Thimi, Bhaktapur.

4.2.5 Data collection and analysis

Standard questionnaires were prepared and used to collect the data on personal information (Age, Sex, Case type etc. as given in Appendix I). SPSS version 11.5 (Statistical Package for Social Science) was used for analysis of data.

4.3 Laboratory methodology

4.3.1 Specimen collection

In tuberculosis diagnosis attention needs to be focused on the problem of microscopy, while an often overlooked problem is that of obtaining adequate specimen. Since correct collection and transport of specimen to the laboratory are important to ensure that the results are accurate and reliable, the patients were given clear instructions.

Aerosols containing tuberculosis bacteria may be formed when the patient coughs to produce a sputum specimen, therefore the patients were instructed to produce specimen either outside in the open air or away from other people and not in confined spaces such as toilets.

4.3.2 Collection of sputum sample

Because TB lesions in the lungs may drain intermittently, it is possible for a specimen to be negative on one day and positive the next (WHO, 1998). For this reason, three specimens were collected for diagnosis as follows:

- One spot specimen when the patient fist presents to the health service.
- One early morning specimen (preferably the next day).
- One spot specimen when the early morning specimen is submitted for examination.

The sputum was collected in wide mouth, transparent, plastic, sterile, leak proof, screw capped container. About 4 ml, mucopurulent or blood stained sputum was collected.

4.3.3 Evaluation of sputum

Collected sputum samples were evaluated using standard protocols (WHO, 1998). When a sputum specimen was being collected, adequate safety precautions were taken to prevent the spread of infectious organisms. The container was labeled and filled in a request form available in the hospital. The specimen was processed within 2 hours or if not processed kept at 4°C. The collected specimen was first macroscopically examined and then stained by Fluorochrome method for microscopy. The sputum samples positive for *M. tuberculosis* were cultured.

4.3.4 Macroscopic examination of sputum sample

Macroscopic evaluation of sputum samples was done using standard methodology (Cheesbrough, 2002). The sputum sample was described macroscopically as given in Table 5.

Table 5. Macroscopic examination of sputum sample

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

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

4.3.5 Microscopic examination of sputum

Microscopic examination of sputum was done using standard methodology (WHO, 1998).

4.3.5.1 Sputum smear preparation

A small portion of appropriate portion of the specimen was selected and transferred on a clean, grease free slide for the smear preparation. The smear was prepared in Biological safety cabinet. Detailed guideline for the sputum smear preparation is mentioned in Appendix V.

4.3.5.2 Acid-fast staining by Fluorochrome method

Since fluorescence microscopy use a low magnification objective to scan smears, allowing a much larger area of the smear to be seen and resulting in more rapid examination, it was used as primary staining procedure. The smear was stained and observed under fluorescence microscope which gives tubercle bacilli as rod shaped emitting a bright yellow fluorescence against a dark background. The microscopic examination of the smear was quantitated as shown in Table 6.

Table 6. Interpretation of stained smear by fluorescence microscopy (WHO, 1998)

<table>
<tr><th rowspan="2">Range</th><th rowspan="2">Report</th><th colspan="3">Fluorescence microscopy magnification</th></tr>
<tr><th>250X</th><th>450X</th><th>630X</th></tr>
<tr><td>0</td><td>No AFB seen</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>1-9/100 fields</td><td>Report exact count</td><td rowspan="4">Divide observed count by 10</td><td rowspan="4">Divide observed count by 4</td><td rowspan="4">Divide observed count by 2</td></tr>
<tr><td>10-99/100 fields</td><td>+1</td></tr>
<tr><td>1-10/ field</td><td>+2</td></tr>
<tr><td>>10/ field</td><td>+3</td></tr>
</table>

The composition and preparation of staining reagents for fluorescence microscopy is mentioned in Appendix III. The Fluorochrome-staining procedure is described in Appendix VI.

4.3.6 Culture of sputum

The definitive diagnosis of mycobacterial disease demands that the causative agent be recovered on culture medium and identified by using differential in vitro tests. Florescence-smear microscopy revealing positive for AFB in sputum samples were cultured on primary culture medium-2% modified Ogawa medium. The composition and preparation of media for culture is mentioned in Appendix III.

4.3.6.1 Homogenization and decontamination of sputum for culture

Sodium hydroxide (Modified Petroff) method was used for the homogenization and decontamination of sputum samples for culture. The composition and preparation of digestion and decontamination reagent is given in Appendix III. Detailed procedure of NaOH (Modified Petroff) method is mentioned in Appendix VII.

4.3.6.2 Culture of homogenized and decontaminated sample

One drop of sediment from homogenized and decontaminated sample was inoculated on each of two culture tubes with the help of sterile Pasteur pipette. The caps of inoculated media were kept loose and incubated at 37°C. When the surfaces of media were dry after few days, the caps were tighten and further incubated for up to 8 weeks. A tube was also incubated at 25°C for up to 8 weeks.

4.3.6.3 Culture examination

All the cultured tubes were examined after one week to detect any rapidly growing mycobacteria which might be mistaken for *M. tuberculosis*. The growth on the media was observed at every week after inoculation. The negative cultures were discarded only after 8 weeks.

4.3.6.3.1 Recording and reporting of laboratory results

A report was sent immediately when the culture was found contaminated so that a repeated specimen could be requested. Since culture reports should be qualitative as well as quantitative, it was reported as shown in Table 7.

Table 7. Recording and reporting of culture (WHO, 1998)

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

4.3.6.3.2 Microscopic examination by ZN-staining

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

4.3.6.3.3 Subculture from Ogawa medium

The growth on Ogawa medium was sub-cultured on L-J medium for screening, biochemical tests, cytochemical staining and antimicrobial susceptibility testing. The

sub-cultured tube was incubated at 37°C for upto 4 weeks or till fine visible growth was observed.

4.3.7 Identification of isolates

Although a presumptive diagnosis was made by cultural morphology and serpentine cord formation, confirmatory tests were carried out by various methods.

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

The growth was screened for *M. tuberculosis* by selective inhibition with PNB in a PNB containing L-J medium.

4.3.7.1.1 Inoculum preparation

One loopful of growth with a 3 mm diameter loop from a sub-cultured L-J medium was mixed with 5 ml of sterile distilled water to give 4 mg/ml suspension of growth.

4.3.7.1.2 Inoculation and incubation

One loopful of the suspension taken with a 3 mm diameter loop was inoculated onto two slants of PNB containing and PNB free media. The slants were incubated at 37°C up to 4 weeks. The slants were observed on 4^{th} day and 4^{th} week of incubation. The preparation of 0.5 mg/ml PNB containing L-J media is given in Appendix III.

4.3.7.2 Cytochemical staining

Cytochemical staining was performed from the sub-cultured tube. Neutral red was used for the cytochemical staining of tubercle bacilli.

4.3.7.2.1 Neutral red test

- 5 ml of 70 %ethyl alcohol was placed in a screw capped tube and was inoculated with few colonies of Mycobacteria from L-J medium.

- The Mac Cartney bottle was incubated at 37°C for 1 hour.
- After incubation, the supernatant alcohol was carefully removed by micropipette and 5 ml of distilled water, and 0.2 ml of 0.05% aqueous solution of neutral red was added.
- The fluid was made alkaline by adding 1/100 N NaOH drop by drop till the color was just amber.
- The bottle was further incubated at water bath for another 1 hour with frequent shaking.
- Any degree of pink or red staining of the colonies suspended in the amber fluid was regarded as positive neutral red test.

The composition and preparation of cytochemical reagents is mentioned in Appendix III

4.3.7.3 Biochemical tests

Biochemical tests were performed from the sub-cultured tube in order to aid in the identification of the isolates. Two different types of catalase tests were performed.

4.3.7.3.1 Drop catalase test

The L-J medium tube having confluent growth was taken and one to two drops of the freshly prepared tween-peroxide mixture was added. It was observed for a period of 5 minutes for the formation of bubbles which indicated positive test (WHO, 1998).

4.3.7.3.2 68°C labile catalase test

68°C labile test can be used instead of Niacin test to differentiate *M. tuberculosis* from other mycobacteria (Fujiki, 2001). The following procedures were followed for the test:

- A bacterial suspension was made by suspending several loopful of growth into the screw-capped tube containing 0.5 ml of 0.067 M phosphate buffer solution.
- The suspension was then incubated in 68°C water bath for 20 minutes.

- Then the suspension was cooled to room temperature.
- 0.5 ml of freshly prepared tween-peroxide mixture was added to the tube and recapped loosely.
- The tube was observed without shaking for the bubble formation. Negative tubes were discarded only after 20 minutes.

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

4.3.8 Antimicrobial susceptibility testing

Antimicrobial susceptibility test was performed by proportion method as recommended by IUATLD and WHO. Each strain was tested against four primary anti-tubercular drugs viz. INH, RFP, SM and EMB.

4.3.8.1 Preparation of drug containing media

The drug containing media were prepared with different concentrations of primary anti-tubercular drugs as shown in Table 8.

Table 8. Concentrations of primary anti-tubercular drugs used in DST

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

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

4.3.8.2 Preparation of bacillary suspension

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

The preparation of Mac Farland No. 1 is mentioned in Appendix III.

4.3.8.3 Dilution of bacillary suspension for inoculation

1 mg/ml bacillary suspension was diluted by serial 10 fold dilutions with sterile distilled water. The dilutions were made as shown in Table 9.

Table 9. Serial dilution of bacillary suspension

	10^{-1}	10^{-2}	10^{-3}
1 mg/ml-bacillary suspension	0.1 ml	0.1 ml	0.1 ml
Sterile distilled water	0.9 ml	0.9 ml	0.9 ml

4.3.8.4 Inoculation and incubation

- From 10^{-1} dilution, eight drug containing media of different concentrations were inoculated with one loopful (0.1 ml) of bacillary suspension.
- Similarly from each dilution, three controls of plain L-J media were inoculated with the respective bacillary suspensions i.e. 10^{-1} dilution in control I, 10^{-2} dilution in control II and 10^{-3} dilution in control III.

- All these tubes were incubated at 37°C for 4 weeks and observed at weekly intervals.

4.3.8.5 Interpretation of results

10^{-3} dilution represents 1% of 10^{-1} dilution. Therefore, any colonies growing on drug containing medium inoculated with the 10^{-1} dilution that equal or more the number of colonies growing on the control medium inoculated with the 10^{-3} dilution represents 1% or more of the test population. The results were thus, interpreted as follows:

4.3.8.5.1 Sensitive (S)

When there was no growth on the culture media with the "critical" concentration of the respective drugs, then it was classified as "sensitive". The critical concentrations of each drug are given in Table 10.

Table 10. Critical concentrations of primary anti-tubercular drugs used in the study

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

4.3.8.5.2 Resistant (R)

The growth on the drug containing medium was compared with the growth on the control at 10^{-3} dilution. When the growth on the culture media with the "critical" concentration of the respective drug was equal or more than on control III, the isolate was classified as "resistant".

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

4.3.9 Quality control

Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. During this study, quality control was applied in various fields (Fujiki, 2001; WHO, 1998; IUATLD, 1998).

- Any leaking and broken specimen containers were discarded by autoclaving and requested for a repeated specimen.
- Smears were prepared only inside the Biological safety cabinet.
- Fresh reagents were used for the staining procedure and other biochemical tests and cytochemical staining.
- Slides were stained in batches with maximum of 12 slides per batch.
- Temperature in all instruments (Inspissator, Water bath, Refrigerator and Incubators) were checked daily and recorded in the chart.
- The inspissator was cleaned after each batch of culture media prepared. Its temperature was also checked during each period of media preparation.
- Those media with faulty coagulation, discoloration or bubbles were discarded.
- While using readymade dehydrated media, the manufacturer’s instructions for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective, inhibitory, and biochemical properties of the media.
- The sputum specimens were processed in batches according to the capacity of centrifuge.
- Each batch of prepared media was checked for sterility by incubating at 37°C for 24 hours.
- All prepared media were kept in the dark in the refrigerator and unused media were discarded after 4 weeks.

- Cross-contamination of culture was avoided by using individual pipettes or loops and strict aseptic techniques.
- Positive and negative controls were used for biochemical and cytochemical tests. *M. tuberculosis* H_{37}Rv was used as positive control for drop catalase test and neutral red test.
- When each newly batch of drug media was prepared, the quality of media was checked by inoculating standard strain *M. tuberculosis* H_{37}Rv suspension onto a series of drug containing media. The detailed procedure for quality check of drug containing media and interpretation table is given in Appendix VIII.

CHAPTER V

5. RESULTS

The study was carried out from September 2006 to June 2007 at National Tuberculosis Centre (NTC), Thimi, Bhaktapur, Nepal so as to screen *Mycobacterium tuberculosis* using inhibitory substance-para-nitrobenzoic acid and, cytochemical staining and drug susceptibility of the isolates to primary anti-tubercular drugs.

A total of 857 suspected patients of pulmonary tuberculosis (PTB), attending NTC was included in this study. All the patients included in the study were tested for acid fast bacilli (AFB) by fluorescence microscopy and those sputum samples showing positive for AFB were further preceded.

This chapter presents the actual findings made during the study period.

5.1 Age and gender of the suspected patients enrolled in the study

Among the studied 857 cases, 585 (68.3%) were male with the highest number in age group 26-30 years (9.2%) followed by the age group 21-25 years (9.0%), and 272 (31.7%) were female with the highest number in age group 21-25 years (6.7%) followed by 26-30 years (4.2%). The age and genderwise of the suspected patients included in the study is shown in Table 11.

The total number of suspected patients enrolled in this study is shown in Figure 2.

Table 11. Age and genderwise distribution of the suspected patients included in the Study

		Gender of the suspected patients				Total	
		Male		Female			
		Count	% of Total	Count	% of Total	Count	% of Total
Age group distribution (in years)	5-10	2	0.2%	1	0.1%	3	0.4%
	11-15	12	1.4%	14	1.6%	26	3.0%
	16-20	54	6.3%	29	3.4%	83	9.7%
	21-25	77	9.0%	57	6.7%	134	15.6%
	26-30	79	9.2%	36	4.2%	115	13.4%
	31-35	73	8.5%	17	2.0%	90	10.5%
	36-40	43	5.0%	28	3.3%	71	8.3%
	41-45	57	6.7%	21	2.5%	78	9.1%
	46-50	55	6.4%	24	2.8%	79	9.2%
	51-55	30	3.5%	14	1.6%	44	5.1%
	55-60	34	4.0%	14	1.6%	48	5.6%
	Above 60	69	8.1%	17	2.0%	86	10.0%
Total		585	68.3%	272	31.7%	857	100.0%

5.2 Pattern of acid fast bacilli in sputum smears by fluorescence microscopy

Out of 857 sputum samples, 611 (71.3%) were negative for AFB by fluorescence microscopy and 246 (28.7%) sputum samples showed AFB on fluorescent staining. Out of total positive cases for AFB, 193 (78.45%) were male and 53 (21.55%) were female. The number and percentage of AFB smears positive and negative cases is shown in Table 12.

Table 12. AFB smears positivity among male and female patients

		Gender of the suspected patients				Total	
		Male		Female			
		Count	% of total	Count	% of total	Count	% of total
Fluorescence staining	Negative	392	45.7%	219	25.6%	611	71.3%
	1+	103	12.0%	27	3.2%	130	15.2%
	2+	54	6.3%	13	1.5%	67	7.8%
	3+	36	4.2%	13	1.5%	49	5.7%
Total		585	68.3%	272	31.7%	857	100.0%

The highest number of smear positive for AFB was observed in age group 21-25 years followed by age groups 31-35 years and 16-20 years respectively. The agewise distribution of AFB smear positive and negative cases by Fluorochrome staining is shown in Table 13. The gender distribution of smear positive and negative cases is shown in Figure 3 and agewise distribution of smear positive and negative cases is shown in Figure 4.

Table 13. Agewise distribution of AFB smears positive and negative cases by Fluorochrome staining

Age group of patients (in years)		Fluorescence staining									
		Negative		1+		2+		3+		Total	
		count	%of total	count	%of total	count	%of total	count	%of total	count	%of total
	5-10	2	0.2%	0	0.0%	0	0.0%	1	0.1%	3	0.4%
	11-15	24	2.8%	1	0.1%	0	0.0%	1	0.1%	26	3.0%
	16-20	53	6.2%	12	1.4%	11	1.3%	7	0.8%	83	9.7%
	21-25	101	11.8%	18	2.1%	11	1.3%	4	0.5%	134	15.6%
	26-30	87	10.2%	16	1.9%	8	0.9%	4	0.5%	115	13.4%
	31-35	59	6.9%	14	1.6%	10	1.2%	7	0.8%	90	10.5%
	36-40	48	5.6%	8	0.9%	10	1.2%	5	0.6%	71	8.3%
	41-45	54	6.3%	17	2.0%	4	0.5%	3	0.4%	78	9.1%
	46-50	57	6.7%	15	1.8%	3	0.4%	4	0.5%	79	9.2%
	51-55	35	4.1%	6	0.7%	1	0.1%	2	0.2%	44	5.1%
	56-60	32	3.7%	7	0.8%	3	0.4%	6	0.7%	48	5.6%
	Above 60	59	6.9%	16	1.9%	6	0.7%	5	0.6%	86	10.0%
Total		611	71.3%	130	15.2%	67	7.8%	49	5.7%	857	100.0%

5.3 Pattern of culture results

All sputum samples positive for acid fast bacilli by fluorescence microscopy (246 samples) were cultured on 2% modified ogawa medium. Out of 246 samples cultured, 214 samples were culture positive with varying degree of growth, 12 samples were contaminated, and 20 samples were culture negative. Out of 214 culture positive

samples, 168 samples were from male and 46 samples were from female. The patterns of cultures are shown in Table 14 and Table 15, and in Figure 5, Figure 6 and Figure 7.

Table 14. Pattern of culture results with respect to fluorescence staining

			Fluorescence staining				Total
			Negative	1+	2+	3+	
	Smear negative cases (not cultured)	Count	611	0	0	0	611
		% of Total	71.3%	0.0%	0.0%	0.0%	71.3%
Culture on Ogawa medium	1+	Count	0	37	7	7	51
		% of Total	0.0%	4.3%	0.8%	0.8%	6.0%
	2+	Count	0	30	10	12	52
		% of Total	0.0%	3.5%	1.2%	1.4%	6.1%
	3+	Count	0	37	43	21	101
		% of Total	0.0%	4.3%	5.0%	2.5%	11.8%
	4+	Count	0	4	0	6	10
		% of Total	0.0%	0.5%	0.0%	0.7%	1.2%
	Contamination	Count	0	7	3	2	12
		% of Total	0.0%	0.8%	0.4%	0.2%	1.4%
	Negative	Count	0	15	4	1	20
		% of Total	0.0%	1.8%	0.5%	0.1%	2.3%
Total		Count	611	130	67	49	857
		% of Total	71.3%	15.2%	7.8%	5.7%	100.0%

Table 15. Genderwise distribution of culture results

			Culture on Ogawa medium							Total
			Smear negative case (not cultured)	1+	2+	3+	4+	Contamination	Negative	
Gender	Male	Count	392	37	40	85	6	10	15	585
		% of Total	45.7%	4.3%	4.7%	9.9%	0.7%	1.2%	1.8%	68.3 %
	Female	Count	219	14	12	16	4	2	5	272
		% of Total	25.6%	1.6%	1.4%	1.9%	0.5%	0.2%	0.6%	31.7 %
Total		Count	611	51	52	101	10	12	20	857
		% of Total	71.3%	6.0%	6.1%	11.8 %	1.2%	1.4%	2.3%	100%

5.4 Growth pattern on LJ medium containing PNB

All the culture positive samples of 2% modified ogawa media (214 culture positive samples) were sub-cultured on L-J media, which gave luxuriant growth. The L-J media containing PNB when inoculated and incubated with growth from sub-cultured L-J media, showed no growth.

Table 16. Result of culture positive samples on L-J medium containing PNB

Gender of the patients	Growth on Ogawa medium			Sub-culture on L-J medium		Growth on L-J medium containing PNB	
	Positive	Negative	Contaminated	Positive	Negative	Positive	Negative
Male	168	15	10	168	0	0	168
Female	46	5	2	46	0	0	46

5.5 Results of biochemical tests

5.5.1 Drop catalase test

All the isolates (214 culture positive samples) gave positive results for drop catalase test by the formation of bubbles.

5.5.2 68°C labile catalase test

All the isolates lost catalase activity when heated at 68°C with no bubbles formation till twenty minutes.

5.6 Result of cytochemical staining

Cytochemical staining performed with neutral red gave positive results for all culture positive isolates (214) i.e. red staining of the colonies suspended in the amber fluid was observed.

Table 17. Results of biochemical tests and cytochemical staining

Gender of the patients	No. of isolates	Drop catalase test		68^0C labile catalase test		Neutral red test	
		Positive	Negative	Positive	Negative	Positive	Negative
Male	168	168	0	0	168	168	0
Female	46	46	0	0	46	46	0
Total	214	214	0	0	214	214	0

5.7 Antibiotic susceptibility pattern of *Mycobacterium tuberculosis*

Among 214 *M. tuberculosis* isolates, 109 (50.9%) were sensitive to INH, 149 (69.6%) were sensitive to RFP, 120 (56.1%) were sensitive to SM and 159 (74.3%) were sensitive to EMB.

Similarly, 105 (49.1%) were resistant to INH, 65 (30.4%) were resistant to RFP, 94 (43.9%) were resistant to SM and 55 (25.7%) were resistant to EMB.

Table 18. Pattern of antibiotic susceptibility of isolates

Antibiotics used	Gender of the patients							
	Male		Male		Female		Female	
	Resistant		Sensitive		Resistant		Sensitive	
	Count	%	Count	%	Count	%	Count	%
INH	80	37.4	88	41.1	25	11.7	21	9.8
RFP	47	22.0	121	56.5	18	8.4	28	13.1
SM	72	33.6	96	44.9	22	10.3	24	11.2
EMB	40	18.7	128	59.8	15	7.0	31	14.5

5.7.1 Resistance pattern of the isolates

Of the 214 tested samples, 121 (56.54%) were found to be resistant to one or more drugs. Resistance to one drug was shown by 11.21% of the isolates. Resistance to two, three and four drugs were shown by 14.95%, 13.55% and 16.82% of the isolates respectively. MDR was found in 62 (28.97%) of the isolates.

Table 19. Pattern of drug resistance of *M. tuberculosis* (n=214) determined by the proportion method.

S. No.	Pattern	No. of strains	
		Count	Percent (%)
1	Resistance	121	56.54
2	Monoresistance to	24	11.21
	INH	11	5.14
	RFP	1	0.47
	SM	12	5.61
	EMB	0	--
3	Resistance to two drugs	32	14.95
	INH+RFP	3	1.40
	INH+SM	24	11.21
	INH+EMB	3	1.40
	RFP+SM	0	--
	RFP+EMB	1	0.47
	SM+EMB	1	0.47
4	Resistance to three drugs	29	13.55
	INH+RFP+SM	15	7.01
	INH+RFP+EMB	8	3.74
	RFP+SM+EMB	1	0.47
	INH+SM+EMB	5	2.34
5	Resistance to four drugs INH+FRP+SM+EMB	36	16.82
6	MDR-TB	62	28.97
	INH+RFP	3	1.40
	INH+RFP+SM	15	7.01
	INH+RFP+EMB	8	3.74
	INH+FRP+SM+EMB	36	16.82
7	Resistance to INH & others	105	49.07
8	Resistance to RFP & others	65	30.37
9	Resistance to SM & others	94	43.93
10	Resistance to EMB & others	55	25.70

5.7.2 Susceptibility pattern of *M. tuberculosis* among different groups

Among different age groups, the highest percentage of resistant was found in 51-55 years age group i.e. 83.33%, followed by 75% in 31-35 years age group and 70% in 46-50 years age group.

Similarly, the highest percentage of sensitive isolates was found in 56-60 years age group i.e. 64.29%, followed by 63.64% in above 60 years age group and 58.33% in 26-30 years age group.

Table 20. Antibiotic susceptibility pattern among different age groups of patients

Age groups (years)	No. of isolates	INH sensitivity		RFP sensitivity		SM sensitivity		EMB sensitivity		Resistant	%	Sensitive	%
		R	S	R	S	R	S	R	S				
5-10	1	0	1	0	1	1	0	0	1	1	100	0	0
11-15	2	2	0	2	0	2	0	2	0	2	100	0	0
16-20	29	13	16	10	19	12	17	9	20	16	55.17	13	44.83
21-25	30	15	15	11	19	13	17	9	21	16	53.33	14	46.67
26-30	24	8	16	5	19	8	16	3	21	10	41.67	14	58.33
31-35	28	19	9	13	15	20	8	12	16	21	75	7	25
36-40	17	8	9	5	12	5	12	4	13	9	52.94	8	47.06
41-45	21	11	10	6	15	7	14	4	17	14	66.67	7	33.33
46-50	20	13	7	5	15	12	8	5	15	14	70	6	30
51-55	6	4	2	4	2	5	1	3	3	5	83.33	1	16.67
56-60	14	5	9	1	13	4	10	1	13	5	35.71	9	64.29
Above 60	22	7	15	3	19	5	17	3	19	8	36.36	14	63.64
Total	214	105	109	65	149	94	120	55	159	121	56.54	93	43.46

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Tuberculosis is the leading cause of death from infectious disease with two billion people infected worldwide. In Nepal, every year 40,000 people develop active TB with 20,000 infectious pulmonary cases. Despite of the adoption of the DOTS strategy by National Tuberculosis Programme (NTP) from 1996, 5,000 to 7,000 people still continue to die each year from TB (NTC, 2007). Much concern has been expressed about multi-drug resistant (MDR-TB) tuberculosis; a worse scenario in HIV infected patients.

In total, 857 sputum samples were collected from the suspected TB patients with 585 (68.3%) male and 272 (31.7%) female patients. The age of the suspected patients in this study ranged from 9 years to 95 years. The highest number of cases was from age group 26-30 years in male and 21-25 years in female.

Out of 857 samples, 246 samples (28.7%) were smear positive for acid fast bacilli (AFB) and 611 samples (71.3%) negative for AFB. The highest number of cases of AFB smear positive was observed in age group 21-25 years with close figures in age groups 31-35 years and 16-20 years. This shows that the maximum number of TB patients is in the productive age group. This finding was concordant with similar studies presented by Chhetri (2001) and Bhattarai (2003).

Since about fifteen times as many fields can be scanned by fluorescence microscopy as by Ziehl-Neelsen microscopy in the same period, the smear positivity rate might have been increased than in similar microscopy results in past studies. According to Toman (1979), fluorescence microscopy yields more true positive, and no more false positive than ZN microscopy.

In 246 smear positive samples for AFB, 214 samples (87%) were positive for culture with varying degree of growth. 20 samples (8.1%) were culture negative though they were smear positive for AFB. A negative culture result with the specimen containing tubercle bacilli may be due to various causes. The causes may be as follows (WHO, 2004; Toman, 1979):

- In patients receiving chemotherapy, the organisms may have lost their ability to grow on culture media and be practically dead.
- In patients who have not had chemotherapy, sputum specimens may have been exposed to sunlight or heat (during transportation by the patients).
- Excessive decontamination (during specimen processing and inoculation).
- Presence of AFB other than *M. tuberculosis.*

4.9% of smear positive samples (12) processed for culture were contaminated. According to WHO (1998), a contamination rate of 2 % to 3% is acceptable in laboratories that receive fresh specimens; but it may be as high as 5% to 10% if specimens (especially sputum) take several days to reach the laboratory. In this study, the contamination rate was slightly higher than the acceptable range which might be due to improper transportation of the sample to the laboratory, inefficient decontamination and other laboratory procedures.

A single test cannot be used to confirm the isolates as *M. tuberculosis.* The buff coloured and rough colonies appearing bread crumbs or cauliflower; no growth in less than one week, and serpentine cord formation, were used as preliminary identification criteria.

PNB (p-$O_2NC_6H_4COOH$) is a carboxylic acid compound that shows appreciable acidity. It contains carboxyl group attached to hydrogen (COOH), and alkyl group (RCOOH). It is stronger acid than benzoic acid.

LJ medium containing PNB has been successfully used as the screening method. The entire isolates positive for culture (214) gave no growth on inoculation and incubation in LJ media containing PNB. Although few colonies were observed on some tubes, they were regarded as negative as recommended by Fujiki (2001). In a similar study carried out by Giampaglia *et al.* (2005), PNB was used successfully in the screening of mycobacteria isolates. Identification of *M. tuberculosis* by using PNB has been adapted in most of the research works and in Mycobacteriology laboratories as well.

Manhadev *et al*. (2001) had also used LJ media containing PNB for the identification of isolates as *M. tuberculosis.* PNB can also be used with other agar medium to screen *M. tuberculosis* (Rastogi *et al.,* 1989) and can also be incorporated in rapid techniques like in the rapid MGIT/PNB method. WHO (1998) has also recommended the use of PNB along with other simple biochemical tests for the identification of *M. tuberculosis.* This work has also shown that LJ medium containing PNB can be effectively used to screen *M. tuberculosis*. The acidic nature of the compound probably is responsible for the inhibition of growth of *M. tuberculosis.* The method is easy, cheaper and reliable.

All the fresh isolates (214) when tested with neutral red, gave positive result. The cells of the isolates (and $H_{37}Rv$ control) took red coloration with the staining solution.

Since none of the conventional methods used in the Mycobacteriology laboratory can be implemented to identify virulent strains of *M. tuberculosis*, cytochemical staining with neutral red is the simple and rapid technique that can differentiate virulent and avirulent strains of *M. tuberculosis.* The virulent strains fix neutral red in its anionic form in alkaline environment. Tarshis (1962) used neutral red test for distinguishing human tubercle bacilli from other mycobacteria. In similar studies carried out by Hughes *et al.* (1954), Buchanan (1955), Ganguli (1957), Koch *et al.* (1960) and Soto *et al.* (2002), neutral red test was used to distinguish virulent and avirulent strains of *M. tuberculosis*, and to study virulence related factors. A cell wall methyl-branched lipid has been shown to be probably responsible for virulence but no clear explanation of this cytochemical

staining has been obtained, and the component (s) responsible for neutral red staining of virulent *M. tuberculosis* are not known with any certainty (Soto *et al.,* 2002).

This study has also shown that the virulent strains of *M. tuberculosis* stained red with neutral red, the technique which is easy, inexpensive, reliable, and currently capable of being carried out in any laboratory.

Drop catalase test performed on all the isolates (214) was positive with the formation of bubbles of oxygen. Similarly, all the isolates lost their catalase activity when heated at 68°C.

Literature reviews have shown that certain Isoniazid-resistant mutants of *M. tuberculosis* do not pass catalase enzymes. According to WHO (1998) a height of less than 31mm of foam suggest low or no catalase activity by semiquantitative catalase test. Since the whole tube was used for the drop catalase test, the foam observed in case of INH-resistant cases might be due to other INH-sensitive strains present along with the mutant strains.

The catalase tests were performed in order to aid in identification of the isolates as *M. tuberculosis*. 68°C labile catalase test ("Hot catalase test") can be used in place of niacin test to differentiate *M. tuberculosis* from other mycobacteria (Fujiki, 2001). Kent *et al*. (1985) described the test as valuable tool in identifying strains of *M. tuberculosis* with weakly positive or negative niacin test. According to WHO (1998), one or more of the catalase test together with growth at 25°C on LJ medium and growth on LJ medium containing PNB at 37°C can be used for the identification of tubercle bacilli instead of niacin and nitrate tests.

Among the tested samples (214), 56.54% (121) of the isolates were resistant to one or more primary anti-tubercular drugs and 43.46% (93) were sensitive to all four drugs.

50.9% (109) of the isolates were sensitive to INH, 69.6% (149) were sensitive to RFP, 56.1% (120) were sensitive to SM and 74.3% (159) were sensitive to EMB. Similarly, 49.1% (105), 30.4% (65), 43.9% (94) and 25.7% (55) of the isolates were resistant to INH, RFP, SM and EMB respectively.

83.33% resistance was observed in age group 51-55 years followed by 75% and 70% among age groups 31-35 years and 46-50 years respectively. 14.95% of the isolates were resistant to two drugs, 13.55% to three drugs and 16.82% to all four primary anti-tubercular drugs. 28.97% (62) of the isolates were multi-drug resistant (MDR).

The resistance rate was higher in the study because most of the isolates were from relapse, after defaulted, treatment failure and chronic cases (case types not shown in result section). Stringent statistical methods, strict interrogation procedures and carefully designed protocol with strict adherence, must be followed to categorize different cases of tuberculosis (Toman, 1979).

“Acquired” or “secondary” resistance results due to incorrect chemotherapy. Primary resistance is due to infection with a strain originating from another patient who has acquired resistance owing to inadequate chemotherapy. Chhetri (2001) reported 100% acquired MDR cases. According to Malla (1996), the acquired MDR-TB cases are quite high ranging from 9.6% to 66.7%. In Nepal, MDR-TB in retreatment cases was 20.5% in 2004 (WHO, 2006). Alarming rate of drug resistance has been reported from developing countries like ours, which are mainly acquired resistance. It has been difficult to classify TB cases due to unresponsive nature of the patients and lack of strict and carefully designed protocols.

Among the tested antimicrobial agents, EMB was the most effective drug followed by RFP. EMB is effective against drug-resistant strains of *M. tuberculosis*, with bacteriostatic effect. Similarly, RFP is active against both drug sensitive and resistant strains of *M. tuberculosis* (Toman, 1979). Literature reviews and the present study clearly showed that both EMB and RFP are most effective drugs where higher

percentage of susceptibility is observed. Higher rates of resistance to INH and SM might be due to the fact that because of their low cost and wide spread use in the treatment of TB.

6.2 Conclusion

In conclusion, this study revealed that PNB added to the culture media could be used for the screening of *M. tuberculosis*. All the isolates showed no growth when inoculated and incubated on LJ media containing PNB. So, a simple, low-cost test using growth inhibitors may be incorporated into the culture media enabling differentiation of *M. tuberculosis* and atypical mycobacteria.

The screened isolates were further confirmed as *M. tuberculosis* by biochemical tests. Neutral red test was positive for all the isolates which showed that all the isolates were virulent.

The resistance rate was higher, with higher MDR-TB cases. Out of four primary anti-tubercular drugs used for drug susceptibility testing, EMB and RFP were more effective than INH and SM.

CHAPTER VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

This study was carried out on "screening of *Mycobacterium tuberculosis* by selective inhibition with para-nitrobenzoic acid, its cytochemical staining and drug susceptibility to primary anti-tubercular drugs" at National tuberculosis Centre, Thimi, Bhaktapur, Nepal. The objective of the study was to develop a test using PNB added to culture medium to screen *M. tuberculosis*, its virulence test and drug susceptibility pattern of the isolates.

A total of 857 samples were taken in the study period. Out of total, 585 samples were from male and 272 samples from female.

The results of the study revealed following findings:

- 28.7% (246) of the total samples were positive for AFB by fluorescence microscopy; out of which 78.45% (193) were male and 21.55% (53) were female.
- The highest number of AFB smear positive was observed in the age group between 16 years and 35 years.
- 87% (214) of the smear positive cases for AFB were positive for culture; 8.1% (20) were culture negative and 4.9% (12) were contaminated.
- There was no growth of the isolates when inoculated and incubated in LJ media containing PNB.
- The drop catalase test was positive for all the isolates.
- When 68°C labile catalase test was performed, all the isolates lost their catalase activity at that temperature with no bubbles formation till 20 minutes.

- All the isolates were virulent as they gave positive results with the cytochemical staining by using neutral red.
- Antimicrobial susceptibility test was performed with primary sets of anti-tubercular drugs.
- Of the total 214 samples tested for drug susceptibility, 56.54% (121) were resistant to one or more drugs whereas 43.46% (93) were sensitive to all four drugs.
- The highest resistance was observed with INH i.e. 49.1% (105) followed by SM (43.9% i.e. 94), RFP (30.4% i.e. 65) and EMB (25.7% i.e. 55).
- 28.97% (62) of the isolates were multi-drug resistant.
- The highest percentage of resistance i.e. 83.33% was observed in age group 51-55 years; followed by 75% and 70% in age groups 31-35 years and 46-50 years respectively.
- EMB and RFP were the most effective drugs in comparison to INH and SM.

7.2 RECOMMENDATIONS

On the basis of the findings made by this study, following recommendations are put forward:

- Test using LJ media containing PNB is useful for the screening of *Mycobacterium tuberculosis* in diagnostic mycobacteriology.
- Cytochemical staining with neutral red is useful for the virulence test of the fresh laboratory isolates of *M. tuberculosis*.
- One or more catalase tests combined with no growth on LJ media containing PNB is useful for the definitive diagnosis of *M. tuberculosis* instead of other tedious tests.
- Semiquantitative catalase activity may be an indicator of isoniazid-resistant strains.

CHAPTER-VIII

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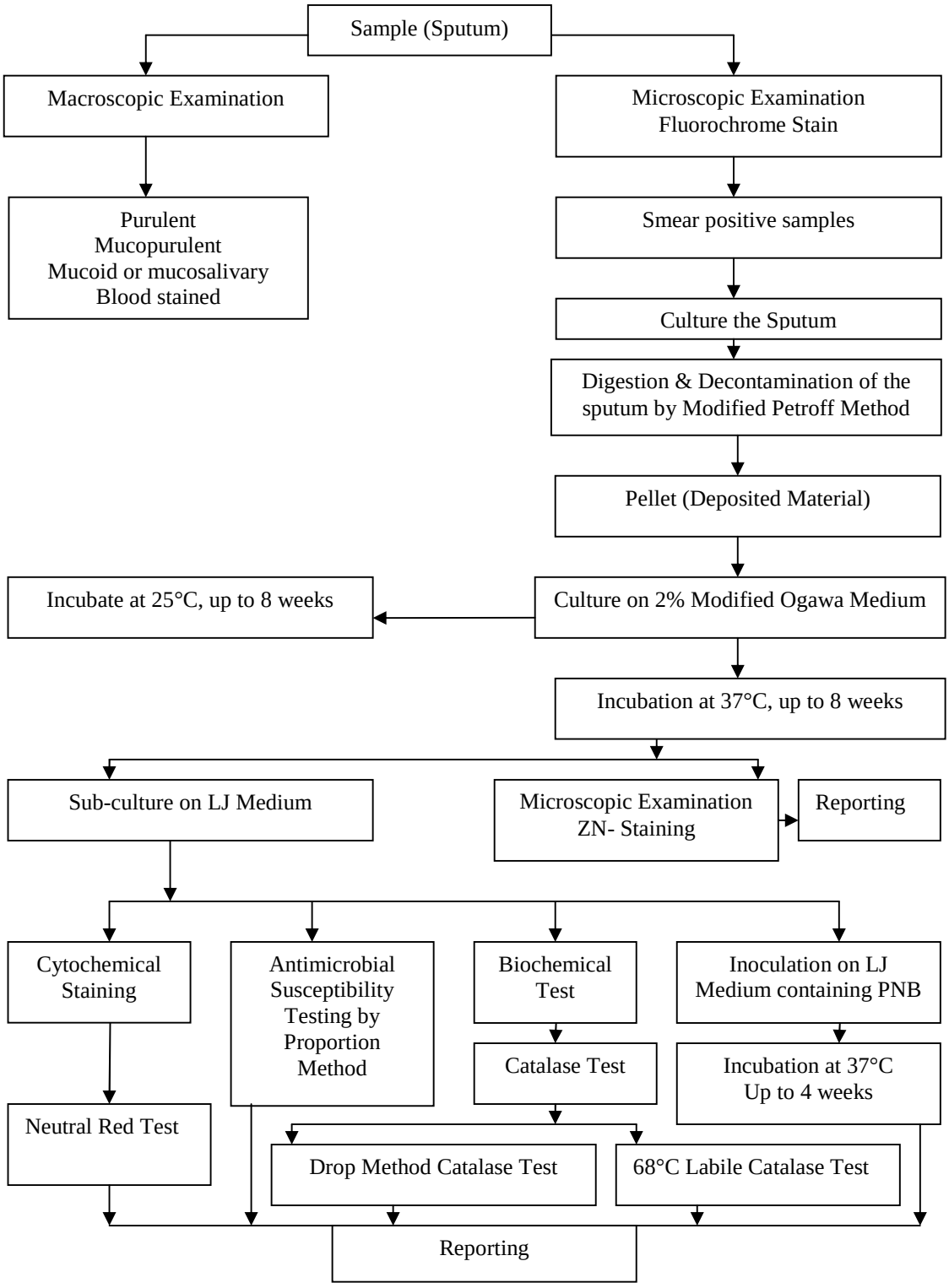
Sample (Sputum)
Macroscopic Examination
Microscopic Examination
Fluorochrome Stain
Purulent
Mucopurulent
Mucoid or mucosalivary
Blood stained
Smear positive samples
Culture the Sputum
Digestion & Decontamination of the sputum by Modified Petroff Method
Pellet (Deposited Material)
Incubate at 25°C, up to 8 weeks
Culture on 2% Modified Ogawa Medium
Incubation at 37°C, up to 8 weeks
Sub-culture on LJ Medium
Microscopic Examination
ZN- Staining
Reporting
Cytochemical
Staining
Antimicrobial
Susceptibility
Testing by
Proportion
Method
Biochemical
Test
Inoculation on LJ
Medium containing PNB
Catalase Test
Incubation at 37°C
Up to 4 weeks
Neutral Red Test
Drop Method Catalase Test
68°C Labile Catalase Test
Reporting

APPENDIX-I

Questionnaire

Lab No. : ……. / S. No. :…….. Date: ……

Name: ……... Age: …… Sex: M / F

Address: ……...

Case type: New Relapse Chronic Follow up After defaulted

Examination: Culture Sensitivity

Macroscopic examination: Purulent Mucopurulent

Mucoid or mucosalivary Blood stained

Smear: Positive Exact count…… +1 +2 +3

Negative

Culture: Positive Exact count…… +1 +2 +3 +4

Negative Contamination

Growth on LJ medium containing PNB: Yes No

Biochemical and Cytochemical staining:

Catalase test i) Drop method Positive Negative

ii) 68°C labile method Positive Negative

Neutral red test Positive Negative

Antimicrobial susceptibility testing:

	INH µg/ml		RFP µg/ml		SM µg/ml		EMB µg/ml	
	0.25	8.0	20.0	**40.0**	**4.0**	8.0	1.0	**2.0**
Interpretation								

Control: I

: II

: III

Critical concentrations

INH 0.25 µg/ml

RFP 40.0 µg/ml

SM 4.0 µg/ml

EMB 2.0 µg/ml

APPENDIX II

Materials used during the study

1. Equipments

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

2. Glass wares

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

3. Plastics

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

4. Safety

Biological safety cabinet, BSC II A
Discard pans
Protective clothing's
Centrifuge safety cups
Disinfectant
Safety pip petting device

5. Chemicals and media base

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

6. Miscellaneous supplies

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

APPENDIX III

I. Composition and preparation of culture media

A. 2% Modified Ogawa medium

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

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

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

ii. Glycerol, reagent grade

Glycerol was added when the salt solution was warm.

iii. 2% malachite green solution

Malachite green dye	2.0 g
Distilled water	100.0 ml

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

Preparation of whole egg homogenate

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

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

Preparation of complete medium

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

B. Lowenstein-Jensen (L-J) medium

i. Salt solution of L-J medium

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

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

ii. Glycerol, reagent grade 12.0 ml

Glycerol was added when the salt solution was warm.

Preparation of whole egg homogenate

Fresh hen's eggs were cleaned by brush and soap, and then run with tap water. Then dried and wiped the outer surface with spirit cotton. After drying, the eggs were cracked

with a sterile forceps into a Petri dish to check the freshness of the egg. The chalazae attached to the egg yolk were removed and the eggs were transferred to sterile blender. The whole egg homogenate was then filtered through two layers of sterile gauze into a sterile cylinder.

Preparation of complete medium

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

II. Composition and preparation of staining reagents

A. Fluorochrome staining reagents

i. Auramine solution

Auramine	1.0 g
Ethanol	100.0 ml

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

ii. Phenol solution

Phenol melted	30.0 ml
Distilled water	870.0 ml

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

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

iii. Decolorizing solution (20% Sulphuric acid)

Concentrated H_2SO_4	200.0 ml
Distilled water	800.0 ml

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

iv. Counter stain solution (Methylene blue)

Methylene blue	1.0 g
Distilled water	1000.0 ml

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

B. Ziehl-Neelsen staining reagent

i. Fuchsin

Basic fuchsin	3.0 g
Ethanol	100.0 ml

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

ii. Phenol solution

Phenol melted	5.0 ml
Distilled water	95.0 ml

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

Working solution

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

iii. Decolorizing solution (25% Sulphuric acid)

Concentrated Sulphuric acid	250.0 ml
Distilled water	750.0 ml

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

iv. Counter stain solution (Methylene blue)

Methylene blue	1.0 g
Distilled water	1000.0 ml

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

III. Preparation of 0.5 mg/ml para-nitrobenzoic acid (PNB) containing medium

25 mg/ml PNB solution

PNB	250.0 mg
Propylene glycol	10.0 ml

PNB was dissolved in propylene glycol by vortexing till clear solution was obtained.

Complete PNB containing medium

2.0 ml of 25 mg/ml PNB solution was added into 98.0 ml of complete raw L-J medium and mixed well. 5.0 ml of PNB containing L-J medium was dispensed in each tube, then inspissated all the tubes by keeping on a slanted bed, at 90°C for 50 mins. The tubes were cooled, sterility checked and stored in the refrigerator.

IV. Digestion and decontamination reagent

i. 4% Sodium hydroxide solution

Sodium hydroxide, pellets	4.0 g
Distilled water	100.0 ml

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

ii. Sterile saline

Sodium chloride, pellets	0.85 g
Distilled water	100.0 ml

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

V. Composition and preparation of Biochemical and Cytochemical reagents

i. Catalase reagents

0.067M phosphate buffer solution, P^H 7.0

Anhydrous disodium phosphate	9.47 g
Distilled water	1000.0 ml

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

Monopotassium phosphate	9.07 g
Distilled water	1000.0 ml

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

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

Tween 80 (10%)

Tween 80	10.0 ml
Distilled water	90.0 ml

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

Complete Catalase reagent (Tween-peroxide mixture)

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

ii. Neutral red reagents

0.01N sodium hydroxide solution

Sodium hydroxide pellets (analytical grade)	0.4 g
Distilled water	1000.0 ml

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

0.05% Neutral red solution

Neutral red	0.05 g
Distilled water	100.0 ml

Neutral red was dissolved in distilled water by frequent shaking and stored at room temperature.

VI. Mc Farland No. 1 preparation

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

APPENDIX IV

Preparation of Drug solutions and Drug containing media

Primary anti-tubercular drugs used

Name of drugs	**Code**	**Company**
Isonicotinic acid hydrazide	INH	Sigma
3-(4-Methyl piperazinyliminomethyl) rifamycin sv	RFP	Sigma
Streptomycin sulphate	SM	Sigma
Ethambutol dihydrochloride	EMB	Sigma

1. Isoniazid (INH)

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

Concentration in drug media (µg/ml)

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

2. Rifampicin (RFP)

Solution I: 20.8 mg RFP mixed with 5.0 ml Propane-1, 2 diol (4000 µg/ml)
Solution II: 4.0 ml of Solution I added up to 50.0 ml distilled water (400 µg/ml)
Solution III: 20.0ml of Solution II added up to 40.0ml distilled water (200 µg/ml)

Concentration in drug media (µg/ml)

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

3. Streptomycin (SM)

Solution I: 21.3 mg SM mixed with 4 ml distilled water (4000 µg/ml)
Solution II: 1.0 ml of Solution I added up to 50.0 ml distilled water (80 µg/ml)
Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (40 µg/ml)

Concentration in drug media (µg/ml)

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

4. Ethambutol (EMB)

Solution I: 34.0 mg EMB mixed with 25.0 ml distilled water (1000 µg/ml)
Solution II: 1.0 ml of Solution I added up to 50.0 ml distilled water (20 µg/ml)
Solution III: 20.0 ml of Solution II added up to 40.0 ml distilled water (10 µg/ml)

Concentration in drug media (µg/ml)

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

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

APPENDIX V

Sputum smears preparation (WHO, 1998; CDC 1985)

The method of smear preparation most used in developing countries and in those laboratories where a rapid smear examination is required, is the direct smear. The cheesy, necrotic, blood tinged particles in the specimen was selected because they are the most likely to produce positive smear results. As a rule of thumb, the thickness of a smear should be such that a newspaper can be read through the smear out on the slide. The following steps are involved in the sputum smear preparation:

1. A new, clean, unscratched, grease free slide was taken and labeled at one end with the relevant patient number.
2. An appropriate portion of the specimen was transferred to the slide by using an applicator stick. Blood specked, opaque, grayish or yellowish cheesy mucus was used for smear preparation whenever it was present.
3. The specimen was smeared on the slide with the broken end of the stick over an area approximately 20 mm by 10 mm. The smear was made thin enough to b e able to read through it. Only one smear was prepared per slide.
4. The smear was air dried for about 15 minutes. Direct heat was not used for drying.
5. The smear was fixed to the slide by passing it through a flame three or four times with the smear uppermost. Then it was cooled before staining.
6. The slide was stained by either fluorescence method or Ziehl-Neelsen method, as per the requirement.

APPENDIX VI

Staining procedures

1. Fluorochrome staining procedure (WHO, 1998)

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

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

i. The heat fixed slides were placed on a staining rack in batches (maximum 12), with smears facing up.
ii. The slides were flooded with carbol fuchsin covering the whole surface.
iii. The slides were heated with spirit cotton till steam comes off from the stain. The slides were not allowed to dry.
iv. The slides were left for about 5 minutes.
v. The process was repeated 2-3 times and allowed to stand for few minutes.
vi. The slides were tilted to drain off excess stain. The staining solution was washed off with a gentle stream of running water.

vii. The slides were tilted to drain off excess water. Then, the slides were decolorized with 20% Sulphuric acid, until solution runs clear.

viii. The slides were then washed with a gentle stream of running water and again tilted to drain off excess rinsed water.

ix. 0.1% Methylene blue was poured to cover the whole surface of the slides and left for 2-3 seconds.

x. Methylene blue was then poured off and the slides were again washed with a gentle stream of running water.

xi. The slides were tilted and placed on the slide rack to dry.

xii. One drop of immersion oil was put on the stained smear and examined under 100X objective with 10X eye piece lens.

APPENDIX VII

Sodium hydroxide (Modified Petroff) method for digestion and decontamination of sputum sample (Fujiki, 2001; IUATLD 1998)

All currently available digesting and decontaminating agents are to some extent toxic to tubercle bacilli; therefore, to ensure the survival of the maximum number of bacilli in the specimen, the procedure must be precisely followed. The success of homogenization and decontamination depends on:

- The greater resistance of bacilli to strongly alkaline or acidic digesting solutions.
- The length of exposure time to these agents.
- The efficiency of the centrifuge used to sediment the tubercle bacilli.

First devised by Petroff, this method is widely used in most of the developing countries. The technique of Petroff to decontamination with up to final maximum concentration of 2% NaOH is given preference worldwide. The 4% NaOH solution represents the upper limit of its concentration. (Petroff himself suggested 3% for sputum specimens). The NaOH decontamination is harmful to mycobacteria: extending the time of contact will kill an increasing proportion of tubercle bacilli in the specimen. Therefore, the NaOH concentration is increased (up to 4% maximum), and never the exposure time. The following steps are involved in this method:

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

APPENDIX VIII

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

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

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

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

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

APPENDIX IX

Identification of *Mycobacterium tuberculosis*

Since no single test can differentiate *M. tuberculosis* from other mycobacteria, a battery of tests, along with the colony morphology enables an identification of *M. tuberculosis* strains.

The following schemes can be used for the identification of *M. tuberculosis*

- Slow growth rate with growth temperature of 35-37°C only.
- Eugonic colonies with no pigmentation.
- Loss of catalase activity at 68°C.
- Neutral red test positive.
- No growth on L-J medium containing PNB.

Differentiation of Mycobacteria

S No.	Characteristics	*M. tuberculosis*	NTM
1	Colony morphology	Rough, eugonic	Mostly smooth
2	Growth at 37°C	+	+/-
3	Growth at 25°C	-	+
4	Pigmentation	-	+/-
5	Growth on L-J medium containing PNB	-	+
6	68°C labile catalase test	-	+