CHAPTER I

1. INTRODUCTION

Mycobacterium is a single genus of Mycobacteriaceae family, with G+C content of 61-71% in DNA, similar to that of other mycolic acid producing bacteria *Nocardia* (60-69%), *Rhodococcus* (54-69%) and *Corynebacterium* (51-59%), except *M. leprae* (54-57%) (Nolte & Metchock, 1995). The genus *Mycobacterium* consists of prokaryotic, rod shaped organisms that at some stage of their growth cycle possess the distinctive property of acid fastness during staining (Madigan & Martinko, 2006). The Mycobacteria are slightly curved or straight bacilli, 0.2 to 0.6µ by 1 to 10µ in size, sometimes with branching to give filamentous or mycelium like growth but it easily fragments into rods or coccoid elements. Mycobacteria are not readily stained by the Gram method but are considered gram positive. Acid fastness may be partly or completely lost at some stage of growth by some proportion of the cells of some species and cells of rapidly growing mycobacteria may be less than 10% acid fast. The mycobacteria are aerobic, non spore forming and non motile bacilli with variable colony morphology among species. The generation time range from 2 to > 20 hour among species to produce colonies after 2 days to 8 weeks under optimal incubation conditions. Diffusible pigment is rare, but colonies of some species are regularly or variably yellow, orange, or rarely pink (Nolte & Metchock, 1995).

There are currently more than 100 recognized or proposed *Mycobacterium* species which are naturally divisible into two groups, viz. relatively rapidly growing species whose colonies appear in 7 days or less and slowly growing species whose colonies appear in more than 7 days, on solid media after plating sufficiently dilute suspensions to give well separated colonies under ideal culture conditions (Collier et al., 1998; Nolte & Metchock, 1995). Mycobacteria may be classified as obligate pathogens, facultative or opportunist, or free living saprophytes, based on their ability to multiply in various environment but many previously considered nonpathogenic species are found to be associated in disease in immunocompromised patients.

Mycobacterium tuberculosis complex is a group of closely related species, comprising *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti*, *M. canettii*, *M. caprae* and *M. pinnipedii* which causes tuberculosis in human and animals (Palomino et al., 2007). *M.*

tuberculosis is a human tubercle bacillus and accounts for majority of human pulmonary tuberculosis and extra pulmonary tuberculosis cases. *M. bovis* is a bovine tubercle bacillus responsible for tuberculosis in various animals including human. *M. bovis* BCG is the attenuated strain derived from *M. bovis* which is used as vaccine against tuberculosis in many parts of the world. *M. africanum* is a heterogeneous group of strains of human origin responsible for tuberculosis principally in equatorial Africa (Collier et al., 1998). *M. africanum* may represent an intermediate form between *M. tuberculosis* and *M. bovis*, and its retention as a distinct species is probably not justified (Nolte & Metchock, 1995). *M. microti* is the vole tubercle bacillus and differs from other members of the complex in its curved cell morphology, extremely slow growth rate in vitro and distinct host specific pathogenicity for laboratory animals (Frota et al., 2004). *M. caprae* cause TB preferably in goats and cattle and occasionally in human and wild life species like red deer or wild boar (Aranaz et al., 1999). *M. pinnipedii* cause TB in sea lions and fur seals and occasionally in human (Palomino et al., 2007).

Non Tuberculous Mycobacteria (NTM) are the large number of mycobacterial species, other than tubercle bacilli, frequently found in environmental habitats that may colonize and cause mycobacteriosis in human (Anon, 1989). They may colonize a host without evident tissue invasion and may be isolated coincidentally as contaminants from the environment so they may be incorrectly labeled as the cause of infection. Some NTM are rarely associated with infection whereas others can cause significant pulmonary and extra pulmonary disease in immunocompetent humans, and disseminated infection in immunocompromised hosts (Collier et al., 1998). Runyon classified NTM into four groups, viz. Photochromogens, Scotochromogens, Non photochromogens and Rapid growers, on the basis of their growth rate and pigmentation, which may be useful in identification scheme (Runyon, 1959). NTM are increasingly important causes of clinical infections and increasing prevalence of disease is related both to more intense interactions of humans with certain types of environment and to changes in population demographics, including a growing number of people who are immunosuppressed due to HIV/AIDS, malignancy or medical intervention (Lee et al., 2009). Speciation is particularly important when choosing antibiotic regimens for immunocompromised patient in whom the presence of any acid fast bacilli may be considered clinically significant (Williams et al., 2007).

Leprosy is a chronic granulomatous disease, caused by *M. leprae* (Hansen's bacillus) with anesthetic skin lesions and peripheral neuropathy with peripheral nerve thickening, which may be in tuberculoid or lepromatous form on basis of presence or absence of host cell mediated immune response respectively. The patients with highly bacilliferous leprosy have nasal as well as dermal infection and since *M. leprae* is shed predominantly from nose, leprosy might be an airborne infection (Rees et al., 1977). A patient with untreated lepromatous leprosy can discharge up to 8×10^8 acid fast bacilli in a single nose blow so shedding from nose is more important than shedding from skin lesions for transmission of disease (Nolte & Metchock, 1995). *M. leprae* may be present in sputum but cannot be cultured in vitro.

Mycobacteria should always be identified to the species level, if possible, for epidemiological investigation and treatment. The identification should be based on as many observations as possible, but it is necessary to select only the key biochemical tests that appear to be useful for species suspected (Nolte & Metchock, 1995). The traditional methods, using characters like growth rate, colony morphology, pigmentation, and biochemical tests, are well established, standardized, and relatively inexpensive but slow in providing clinically relevant information whereas newer laboratory methods such as chromatographic analysis and nucleic acid probes have decreased the mycobacteriologist's reliance on biochemical profiles (Forbes et al., 2002).

In treatment of Tuberculosis, first line drugs used are Isoniazid, Rifampicin, Pyrazinamide, Ethambutol and Streptomycin whereas second line drugs like Para-amino salicylic acid, Ethionamide, Cycloserine, Capreomycin, Kanamycin, Amikacin, Ciprofloxacin, Ofloxacin and Rifabutin etc. should be used if resistance or toxicity occurs (Grange, 1998). In vitro susceptibility testing should be performed on every first *Mycobacterium tuberculosis* isolates from patients. Susceptibility testing requires meticulous care in the preparation of medium, selection of adequate samples of colonies, standardization of the inoculums, use of appropriate controls and interpretation of results. The direct susceptibility testing method uses smear positive concentrate containing more than 50 acid fast bacilli per 100 oil immersion fields as an inoculum which provides rapid results but method is less standardized and contamination may occur. Indirect susceptibility testing method uses culture as the inoculum's source. The conventional methods used to determine drug susceptibility of *Mycobacterium tuberculosis* isolates are

absolute concentration, resistance ratio, proportion and BACTEC system, whereas new methods used to test drug susceptibility use genotypic assays using PCR amplification of gene and identification of mutation, high density DNA probe assay, luciferase reporter mycobacteriophage assay (Forbes et al., 2007). Strains of *Mycobacterium avium* complex are intrinsically resistant to anti tuberculosis drugs and many other antimicrobial agents owing to failure of these drugs to penetrate the lipid rich cell wall (Hornick & Schlesinger,1998). Almost all strains of rapidly growing mycobacteria are resistant to anti tuberculosis drugs. The antimicrobial agents used often depend on identification of isolate and result of drug susceptibility studies (Nolte & Metchock, 1995).

Tuberculosis is an infectious disease responsible for huge human casualties, known since antiquity and still posing challenges to human population in 21st century with MDR and XDR TB. Tuberculosis is one of the major public health problem in SAARC region with almost 50% of adults population already been infected with *M. tuberculosis* and are at risk of developing tuberculosis disease (STC, 2010). HIV is the most important fuelling factor for tuberculosis and tuberculosis is the leading cause of HIV related morbidity and mortality. HIV increases the person's susceptibility, by 30 to 50 times, to infection with *M. tuberculosis* and progression of infection to active disease (STC, 2010). According to STC (2008), out of total population 26,284,018 in Nepal, estimated cases were 43,806, but notified cases were 33,439 of all cases of tuberculosis in 2007 with case detection rate 72.4% (all types) and cure rate of 86% in 2006. WHO estimates prevalence and incidence of all types of TB in Nepal as around 71,000 and 48,000 respectively. During July 2009-July 2010 NTP registered 37,732 TB cases, among which 18,681(49.5%) were sputum smear positive. MDR prevalence was 1% in 1996 and increased to 2.9% in 2006 among new cases and 11.7% among retreatment cases in 2006. About 5% of all MDR cases registered are XDR (NTC, 2011).

There are very few studies based on classification of *Mycobacterium* species isolated from sputum and the drug susceptibility pattern of isolates and more studies at tertiary care centre are necessary. The exact speciation of *Mycobacterium* is necessary for differentiation of disease as pulmonary TB or pulmonary mycobacteriosis and commencement of appropriate treatment.

CHAPTER II

2. OBJECTIVES OF THE STUDY

2.1. General objective

To describe the *Mycobacterium* species present in sputum of suspected pulmonary tuberculosis patients and assess drug susceptibility pattern of the isolates.

2.2. Specific objectives

i. To distribute *Mycobacterium* species by observing growth rate, pigmentation, colony characters and performing biochemical tests.
ii. To differentiate *Mycobacterium tuberculosis* complex from Non tuberculous mycobacteria.
iii. To assess the antibiotics resistance pattern of the isolates.
iv. To assess the association of pulmonary mycobacterial infection with habit of smoking, habit of taking alcohol, family history of tuberculosis and BCG vaccination.

CHAPTER III

3. LITERATURE REVIEW

3.1The genus *Mycobacterium*

Mycobacterium is the single genus in Mycobacteriaceae family and comprises non motile, non sporing, weakly Gram positive, acid- alcohol fast, aerobic or microaerophilic, straight or slightly curved, rod shaped actinobacteria, with G+C content of DNA 61-71 mol% (except *M. leprae* with 54-57 mol%). They are 2-10µm in length and 0.2-0.4µm in breadth to form thin slender rods. *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Corynebacterium* possess mycolic acid in their cell wall which give them characteristic acid- alcohol fastness property. There are more than 100 recognized or proposed mycobacterial species (Forbes et al., 2002).

The mycobacterial species that causes tuberculosis in human or other animals are called tubercle bacilli and are grouped into *Mycobacterium tuberculosis* complex which includes *M. tuberculosis*, *M. bovis*, *M. bovis* Bacilli Calmette Guerin (BCG), *M. africanum*, *M. microti*, *M. canettii*, *M. caprae* and *M. pinnipedi* (Palomino et al., 2007). Other mycobacterial species that do not cause TB are called by several names like Non Tuberculous Mycobacteria (NTM), Atypical Mycobacteria, Anonymous Mycobacteria, Unclassified Mycobacteria, Unknown Mycobacteria, Tuberculoid Mycobacteria, Environmental Mycobacteria, Opportunistic Mycobacteria, Mycobacteria other than tubercle bacilli(MOTT) (Forbes et al., 2002).

Based on phenotypic characters, especially growth rate and pigmentation Runyon (1959) classified NTM into four Runyon groups:

Runyon group I. Photochromogens: They are slow growing NTM (i.e. require more than 7 days to appear as colony on solid media) that produce yellow to orange pigment after 24-48 hours incubation after exposure to the light source, which were not pigmented when grown in dark. Examples: *M. kansasii*, *M. marinum* etc.

Runyon group II. Scotochromogens: They are also slow growing NTM which produce yellow to orange pigment irrespective of whether they are incubated in dark or light. Examples: *M. scrofulacium*, *M. szulgai*, *M. simiae* etc.

Runyon group III. Non photochromogens: They are also slow growing NTM which do not produce pigment at all irrespective of whether they are grown up in light or dark. Examples*: M. avium, M. intracellulare*, *M. haemophilum* etc.

Runyon group IV. Rapid growers: They produce visible colonies in solid media within 7 days of incubation .They may be chromogenic like *M. vaccae*, *M. phlei*, *M. thermoresistible* or non chromogenic like *M. chelonae*, *M. fortuitum*.

Some of these NTM are involved in pulmonary mycobacteriosis while others may be involved in mycobacteriosis of other organs, while still others may be saprophytic at all or are rarely involved in disease (Hornick & Schlesinger, 1998). For an immunocompromised person there may not be any non pathogenic mycobacterial species (Good & Shinnick, 1998).

3.2. Diseases

3.2.1. Tuberculosis (TB)

TB is an infectious disease that is caused by a group of closely related organisms that are grouped up into *Mycobacterium tuberculosis* complex, and is characterized by the presence of tubercles in lungs or other organs (Grange, 1998). TB of lungs is called pulmonary TB, which is the contagious type of TB; whereas TB of nervous system, gastro-intestinal tract, genito-urinary tract, bones etc are called extrapulmonary TB which may be non contagious (STC, 2010). TB is known to mankind since antiquity and has made human to suffer throughout the development upto the 21st century. It is still a major public health problem despite the discovery of its vaccination and effective chemotherapy, because of the emergence of Multi-drug resistant (MDR) and Extensively drug resistant (XDR) strains (WHO, 2010).

TB is transmitted by the infectious aerosol containing droplet nuclei containing *Mycobacterium tuberculosis*. When an open case of TB coughs, sneezes, or talks the droplets are released which contain tubercle bacilli, the droplets get evaporated to form droplet nuclei which are smaller in size and can infiltrate the mucosal and ciliary barrier to reach the alveoli of lung (Park, 2007). When droplet nuclei lodge in the alveoli, the tubercle bacilli are engulfed by alveolar

macrophages in which they multiply to form initial lesion or Ghon focus , some bacilli are carried by phagocytic cells to the hilar lymph nodes where additional foci of infection develops, these comprise primary complex (Grange, 2007). In addition bacilli are seeded by further lymphatic and hematogenous dissemination in many organs and tissues, including other parts of the lungs. *M. tuberculosis* is a pathogen capable of producing both progressive disease and latent infection (Parrish, 1998). *M. bovis* may be acquired through milk and cheese products where the primary complexes involve the tonsils and cervical lymph nodes or the intestine and mesenteric lymph nodes (Grange, 2007). Within about 10 days of infection, antigens of tubercle bacilli are processed by antigen-presenting cells and presented to antigen-specific T lymphocytes, the activated T lymphocytes undergo clonal proliferation and release cytokines notably interferon γ, which together with calcitriol, activate macrophages and cause them to form a compact cluster, or granuloma, around the foci of infection. These activated macrophages are termed epitheloid cells because of their microscopical resemblance to epithelial cells and some of them fuse to form multinucleate giant cells (Grange, 2007). The centre of the granuloma contains a mixture of necrotic tissue and dead macrophages, and because of its cheese like appearance and consistency, is called caseation. Activated human macrophages inhibit the replication of the tubercle bacilli, the macrophage in the granuloma are metabolically very active and consume oxygen, and the resulting anoxia and acidosis in the centre of the lesion probably kills most of tubercle bacilli. Granuloma formation is usually sufficient to limit the primary infection: the lesions become quiescent and surrounding fibroblasts produce dense scar tissue, which may become calcified. Even after successful control of primary TB infection, some bacilli remain in non-replicating or slowly replicating dormant state for rest of life of patient. This infectious latent TB infection state is clinically asymptomatic, and the dormant bacilli may reactivate to cause post-primary TB (Parrish, 1998; Dolin, 1994). Cytokines such as TNF- and IFN- , as well as NO contribute significantly to maintaining infection in the latent state (Arritaga, 2002; Flynn, 1998). Programmed cell death (apoptosis) of bacteria laden cells by cytotoxic T cells and Natural killer cells may also contribute to protective immunity. In many individuals the primary complex resolves but after certain intervals in months or year reactivation of dormant foci of tubercle bacilli or exogenous reinfection leads to post primary tuberculosis which tends to occur in upper lobes of the lungs and the same process of granuloma formation occurs but the necrotic element of the reaction causes tissue destruction and the formation and the formation of large

area of caseation termed tuberculomas (Grange, 2007). Proteases liberated by activated macrophages soften and liquefy the caseous material, and an excess of tumor necrosis factor and other immunological mediators causes the wasting and fever characteristic of the disease. Eventually the lesion erodes through the wall of a bronchus, the liquefied contents are discharged and a well aerated cavity is formed. Once the cavity is formed large numbers of bacilli gain access to the sputum and the patient becomes an infectious or open case and may transmit to others (Grange, 2007).

MDR TB is caused by MDR *M. tuberculosis* strains which are resistant to at least two important primary anti tubercular drugs INH and RMP. XDR TB is caused by XDR *M. tuberculosis* strains which are resistant to at least INH and RMP (i.e. MDR TB), resistant to a fluoro-quinolone and resistant to one or more of the injectable drugs Amikacin, Kanamycin and Capreomycin (WHO, 2006).

Overall one third of the world's population is currently infected with tuberculosis and 5-10% of the infected people become actively sick (WHO, 2011). Twenty-two countries bear 80% of the tuberculosis burden worldwide, and 9 million people become ill with active tuberculosis and nearly 2 million people die each year (WHO, 2010).

3.2.2. Mycobacterioses

Non-tuberculous mycobacteria may colonize and cause infection called mycobacteriosis in human and animals (Anon, 1989). They may colonize a host without evident tissue invasion and be may be isolated coincidently as contaminants from environment. Some NTM are rarely associated with infection, whereas others can cause significant pulmonary and extrapulmonary disease in immunocompetent humans and disseminated infection in immunocompromised hosts (Hornick & Schlesinger, 1998). Mycobacteriosis is becoming more prevalent with the increasing prevalence of immunocompromised hosts. The American thoracic society criteria for the diagnosis of pulmonary mycobacteriosis includes presence of cavitary or non cavitary infiltrates on chest X-ray, acid fast bacilli demonstration on two or more respiratory specimens and/or moderate to heavy growth on culture and absence of other reasonable causes like pulmonary

mycosis and tuberculosis. The NTM most commonly associated with pulmonary mycobacteriosis includes *M. avium* complex, *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. abscessus* etc (Hornick & Schlesinger, 1998).

3.3. Genomics and proteomics of mycobacteria

M. tuberculosis $H_{37}Rv$ genome consists of 4,411,529 bp sequence with high G+C content (65.5%) along most of the genome, with skew in only a few regions. A conspicuous group of genes with a very high G+C content (>80%) code for family of PE (proteins with motif Pro-Glu) and PPE (proteins with motif Pro-Pro-Glu) proteins; few genes with low G+C content (<50%) code for transmembrane proteins. Fifty genes code for functional RNA and a single ribosomal RNA (*rrn*) operon is located at 1.5Mbp from the origin of replication (*oriC* locus). Most eubacteria have more than one *rrn* operon located much closer to the oriC locus to exploit the gene-dosage relationship, but a single rrn operon in relatively distant position from *oriC* has been postulated as a contributing factor for slow growth phenotype of tubercle bacilli (Cole, 1994; Brosch, 2000a). Some of sixteen copies of insertion sequence IS6110 are clustered in insertional hot spots sites and six copies of more stable IS1081. Another 32 different insertion sequences are found in the genome of which seven belong to the 13E12 family of repetitive sequences; other insertion sequences has not been described in other organisms (Cole, 1998b). Two prophages, PhiRv1 and PhiRv2, of similar length are found in genome of *M. tuberculosis* $H_{37}Rv$. The genome of various *M. tuberculosis* strains possess seven potential *att* site for PhiRv1 insertion; which interrupts a repetitive sequence of 13E12 family in *M. tuberculosis* $H_{37}Rv$, but this prophage is deleted or rearranged in other strains(Fleischman, 2002). PhiRv2 is more stable and less variable among strains (Cole, 1999).

In *M. tuberculosis* $H_{37}Rv$ genome, protein is encoded by 3,924 open reading frames (ORF) accounting for 91% of the coding capacity of the genome (Cole, 1998a). A bias is found in the overall orientation of the genes with respect to the direction of replication, with only 59% in the same orientation as that of the replication fork. From the predicted ORFs, all encoded proteins have been classified into 11 broad functional groups.

Table 1: Functional classification of proteins of *M. tuberculosis* $H_{37}Rv$ genome (Fleischman, 2002)

Class	Function	Number of genes
0	Virulence, detoxification, adaptation	99
1	Lipid metabolism	233
2	Information pathways	229
3	Cell wall and cell processes	708
4	Stable RNAs	50
5	Insertion sequences and phages	149
6	PE and PPE proteins	170
7	Intermediary metabolism and respiration	894
8	Proteins of unknown function	272
9	Regulatory proteins	189
10	Conserved hypothetical proteins	1,051

One characteristic of *M. tuberculosis* genome is that it has potential to switch from one metabolic route to another including aerobic (e.g. oxidative phosphorylation) and anaerobic respiration (e.g. nitrate reduction); this flexibility is useful for survival in the changing environments within the human host that range from high oxygen tension in the lung alveolus to microaerophilic/anaerobic condition within the tuberculous granuloma. Another characteristic of the *M. tuberculosis* genome is the presence of genes for synthesis and degradation of almost all kinds of lipids from simple fatty acids to complex molecules such as mycolic acids. In total, there are genes encoding for 250 distinct enzymes involved in fatty acid metabolism, compared to only 50 in *E. coli* genome (Cole, 1999). *M. tuberculosis* genome codes for 13 putative sigma factors and more than 100 regulatory proteins for transcriptional regulation.

M. tuberculosis genome lacks MutS based mismatch repair system, which is overcomed by the presence of nearly 45 genes related to the DNA repair mechanism, including three copies of the *mutT* gene which encodes enzyme that removes oxidized guanines whose incorporation during replication cause base pair mismatch, thus providing replication machinery with very high fidelity (Mizrahi, 1998; Cole, 1999).

M. tuberculosis CDC 1551 genome contains 4,403,836 bp with 65.6 G+C% and 4,186 protein encoding genes. *M. bovis* AF212297C genome contains 4,345,492 bp with 65.6 G+C% and 3,920 protein encoding genes. *M. leprae* genome contains 3,268,203 bp with 57.79 G+C% and 1,604 protein encoding genes. *M. avium paratuberculosis* genome contains 4,829,781 bp with 69.3 G+C% and 4,350 protein encoding genes. *M. smegmatis* genome contains 6,988,209 bp with 67.40 G+C% and 6,897 protein encoding genes (Li, 2005; Marri, 2006).

3.4. Mode of transmission

M. tuberculosis is transmitted basically by the inhalation of the infectious aerosol containing tubercle bacilli which may be present in the droplet nuclei generated from droplets produced from the respiratory tract of infectious cases during coughing, sneezing, talking etc.; or on dust surface in the room where patient stay (Park, 2007). The risk of infection mainly depends on closeness of contact as well as the infectiousness of the source case (Grange, 1998).

M. bovis can be transmitted from the animal to man by respiratory route. Besides it may also be transmitted to man with ingestion of infected raw milk, cream and cheese (Chakraborty, 2003; Grange, 1998).

Pathogenic NTM are basically found in environmental sources which may be transmitted to man with inhalation of infectious aerosol containing NTM. The just colonization of the NTM must be distinguished from infection by clinical, histological or radiological evidence before the treatment ensues. They may colonize a host without evident tissue invasion and may be isolated coincidentally as contaminants from the environment (Hornick & Schlesinger, 1998).

3.5. Pathogenesis

The clinical and histological features of TB are the result of the virulence of the tubercle bacillus and, more critically, the nature and effectiveness of the host's defence mechanisms principally cell mediated protective immune reactions (Grange, 1998). *M. tuberculosis* is a pathogen capable

of producing both progressive disease and latent infection (Parrish, 1998).Tubercle bacilli present in infectious aerosol reaching alveoli are phagocytosed by alveolar macrophages. If bacilli are not destroyed they replicate and kill the cell, so local area of inflammation develops and more phagocytes are attracted. Some bacilli are transported to regional lymph nodes and are engulfed by Antigen Presenting Cell (APC) and mycobacterial epitopes are presented on cell surface by MHC class II to $CD4^+$ T helper cells, which then undergo activation and clonal proliferation and produce cytokines, including IFN γ which activates macrophages that phagocytose mycobacteria (Grange, 2007). Mycobacteria survive within macrophages: they inhibit fusion of phagosome to lysosome; they neutralize reactive oxygen intermediates by means of cell wall lipids including mycosides and lipoarabinomannan and by secreting the enzyme superoxide dismutase; they escape from phagosome and replicate in cytoplasm of cell. Activated macrophages form tuberculous granuloma consisting of compact palisade of activated macrophages many layer thick around the area of infection. These activated macrophages resemble microscopically to epithelial cells, so they are called epithelial cells; some fuse to form multinucleated giant cells. The outer region of the granuloma contains lymphocytes which secrete IFN γ and other cytokines that activate and draw more macrophages to the lesion. Activated macrophages produce cytokine TNF α which maintains the integrity of the granuloma but is also a mediator of tissue necrotizing immunopathology and is responsible for wasting of patients with advanced tuberculosis (Grange, 2007). The macrophages in palisade consume oxygen diffusing into granuloma, so center become anoxic and undergoes necrosis to produce cottage cheese like material, called caseation. The anoxia and free fatty acids in caseous centre makes unfavorable environment to kill tubercle bacilli. The granuloma become inactive, fibroblasts surround them with fibrin which then contracts to form scars which may become calcified. This defense renders the disease the disease quiescent in about 95% of primarily infected persons, but some bacilli may survive in latent state being in metabolically dormant state induced by anoxia or in steady state of multiplication and killing by immune mechanism. Latent TB state is clinically asymptomatic and most active TB cases arise as a result of reactivation of dormant bacilli (Parrish, 1998; Dolin, 1994) Post primary tuberculosis develops only in a minority of those who successfully overcome the primary lesions (Grange, 2007).

3.6. Common anti mycobacterial drugs, mode of action and resistance mechanism

3.6.1. Isoniazid

Isoniazid (isonicotinic acid hydrazide) is a synthetic antimicrobial agent with potent bactericidal activity against tubercle bacilli. It effects on mycolic acid synthesis resulting in increased mycobacterial cell fragility, increased intracellular viscosity, decreased cellular hydrophobicity, and loss of acid fastness. Also it interacts with catalase and peroxidase to produce bactericidal free radicals (Dackett et al., 1980). In addition it appears to interfere with NAD metabolism that leads to pleiotropic effects on energy metabolism and macromolecular synthesis (Davis & Weber, 1977; Winder & Collins, 1969).

katG gene in mycobacterial genome encodes for both catalase and peroxidase enzymes; the deletion of this gene confers resistance to Isoniazid (Zhang et al.1992). Also Isoniazid resistance in mycobacterial genome is related to missence mutation in *inhA* gene (Banerjee et al., 1994). *inhA* encodes a protein that may be a component of mycolic acid biosynthesis pathway.

INH is active against replicating tubercle bacilli but slowly replicating bacilli in the caseous lesions are less readily killed and dormant bacilli are unlikely to be affected. *M. tuberculosis*, *M. xenopi*, *M. kansasii* etc are susceptible to Isoniazid whereas *M. avium*, *M. intracellulare*, *M. marinum*, *M. ulcerans* and all rapidly growing mycobacteria are resistant to it (Inderlied & Salfinger, 1995).

INH is well absorbed when administered perorally or intramuscularly. INH is metabolized in the liver and intestines, primarily by acetylation by an N-acetyl transferase. The adverse drug reactions include infrequent age related hepatitis and even less frequently, peripheral neuropathy, hypersensitivity reactions like fever and rash, and arthralgias (Inderlied & Salfinger, 1995).

3.6.2. Rifampicin

Rifampicin is 3,4-(methylpiperazinyl-iminomethylidene)-rifamycin is active against wide variety of non- acid fast bacteria and acid fast bacteria like tubercle bacilli, *M. leprae*, *M. kansasii*, *M.*

haemophilum and *M. marinum*, but is only variably active against MAC and inactive against rapidly growing mycobacteria. Rifampicin inhibits the prokaryotic DNA dependent RNA polymerase by binding to the β subunit and changing its structure, at the catalytic center of the enzyme. Mammalian RNA polymerase is also inhibited by Rifampicin but only at significantly higher concentrations. The change in β subunit structure of RNA polymerase is primarily due to missense mutation of *rpoB* gene with different amino acid. The RNA polymerase of MAC appear to be susceptible to Rifampicin, but the mutation in *rpoB* gene is rare, and impermeability may be most likely reason for the resistance (Inderlied & Salfinger, 1995).

Rifampicin is well absorbed from the gastrointestinal tract and its concentrations $<0.5\mu g/ml$ are bactericidal for wild type isolates of *M. tuberculosis*, and the drug affects intracellular, slowly replicating bacilli in caseous lesions as well as actively replicating tubercle bacilli in open pulmonary cavities. Adverse drug reactions include gastrointestinal and hypersensitivity reactions; however the major effect is hepatotoxicity and a red-orange discolouration of urine, tears, and other body fluids (Inderlied & Salfinger, 1995).

3.6.3. Streptomycin

Streptomycin as well as other amino-glycosides like Amikacin, Kanamycin are used for treatment of tuberculosis and mycobacteriosis. The primary mechanism of action of aminoglycosides is inhibition of the post to pre translocation step of protein synthesis by blocking the binding of aminoacyl tRNA. The molecular basis of Streptomycin resistance in *M. tuberculosis* result from mutation in the gene that encodes ribosomal protein S12 or the 16S rRNA region that is linked to the S12 protein. In a small number of isolates, streptomycin resistance appeared to be result of point mutation in *rpsL* gene. Adverse drug reactions associated with aminoglycosides include hearing loss, tinnitus, loss of balance, and renal failure (Inderlied & Salfinger, 1995).

3.6.4. Ethambutol

Ethambutol is dextro-2,2'-(ethylenediimino)-di-1-butanol-dihydrochloride which is a potent synthetic antitubercular drug. The MICs of ethambutol against *M. tuberculosis* ranges from 1 to

5µg/ml, but the activity against other mycobacteria is variable. The primary mechanism of action of Ethambutol is a bacteriostatic inhibition of cell wall synthesis, while evidences point to a specific effect on arabinogalactan synthesis. The frequency of mutation to EMB resistance in *M. tuberculosis* is on the order of 10^{-5}. The primary adverse effect associated with Ethambutol is a decrease in visual activity due to optic neuritis which is generally reversible upon discontinuation of the drug (Inderlied & Salfinger, 1995).

3.6.5. Pyrazinamide

It is a synthetic derivative of nicotinamide and is rapidly bactericidal in combination with Isoniazid for replicating form of *M. tuberculosis*, with an average MIC of 20µg/ml; but is inactive against non replicating form of tubercle bacilli and totally inactive against other mycobacterial species, including *M. bovis*, MAC and the rapidly growing mycobacteria. It is active only at a slightly acidic pH of 5.5 to 6, and most likely to be active in acidic environment of phagolysosome. It is proposed that pyrazinamide acts on a population of bacteria that are exposed to an acidic environment as a result of immune activation and its clinical administration during the initial phase of treatment reduces the risk of relapse after six months, suggesting that the early pyrazinamide-susceptible population may contribute to the later pool of mycobacteria that persist during prolonged chemotherapy (Turner et al., 2002). It may be bacteriostatistic or bactericidal depending on the concentration achieved at the site of infection. Pyrazinamide is hydrolyzed in the liver to the active metabolite pyrazinoic acid which is responsible for the effect of the drug. *M. tuberculosis* produces a pyrazinamidase and most pyrazinamide resistant strains lack this enzyme (Inderlied & Salfinger, 1995).

Pyrazinamide is well absorbed from the gastrointestinal tract and widely distributed throughout the body. Hepatotoxicity occurs in small number patients, photosensitivity and rash occur rarely in the patients with its use. Gout is an important contraindication because of hyperuricaemia associated with pyrazinamide therapy (Inderlied & Salfinger, 1995).

3.7. Disease diagnosis

3.7.1 Acid fast staining

Mycobacteria contain long-chain; multiply cross-linked fatty acids called mycolic acids in their cell walls which probably give them characteristic acid fast property. These mycolic acids probably complex with basic dye and due to their affinity with these stains, they resist decolourisation by acids or acid-alcohols. Besides Mycobacteria, species of *Nocardia* and *Rhodococcus* are partially acid fast, *Legionella micdadei* is partially acid fast in tissue, and cysts of *Cryptosporidium* and *Isospora* are distinctly acid fast. Acid fastness is affected by age of colonies, medium of growth and ultraviolet light. Rapidly growing species may be acid fast variable. The visualization of acid-fast bacilli in sputum should be considered only presumptive evidence of tuberculosis or pulmonary mycobacteriosis because environmental contamination or mere colonization by Non tuberculous mycobacteria may occur in respiratory tract. Acid fast stained smears of clinical specimens require at least 10^4 acid fast bacilli per milliliter for detection from concentrated specimens (Forbes et al., 2007).

3.7.1.1. Ziehl Neelsen staining

It requires basic fuchsin dye and phenol as mordant. After application of carbol fuchsin to the smear the slide is heated for better penetration of the stain into the cell wall. Mycolic acids and waxes present in bacterial cell wall complex the basic dye and fail to wash out with mild acid alcohol or acids decolourisation. Phenol is protein denaturant and helps in better penetration of the stain into cell wall. If present typical acid fast bacilli appear as purple to red, slightly curved, short or long, thin slender rods (2 to 10µm in length and 0.2 to 0.4µm in breadth). Some non tuberculous mycobacteria may appear beaded or banded such as *M. kansasii*; or pleomorphic, usually coccoid such as *M. avium* complex (Forbes et al., 2007). The counter stains like methylene blue or malachite green stains non-acid fast organisms. The reporting of acid fast smears is given in Appendix VI.

3.7.1.2. Fluorochrome acid fast staining

Fluorochrome stained acid fast slides with Rhodamine or Rhodamine-Auramine require fluorescent microscope with Ultraviolet light illumination for examination. The fluorochrome dyes complex to the mycolic acids in acid fast cell walls. This method is more sensitive than Ziehl-Neelsen staining, because the fluorescent bacilli stand out brightly against the background and the smear is examined in lower magnifications(250X to 400X) so more fields can be examined in short periods. Specimens screening result in higher yield of positive smears in reduced time of examination. One drawback of fluorochrome staining is many rapid growers may not appear fluorescent with these reagents (Forbes et al., 2007). The reporting of acid fast smears is given in Appendix VI.

3.7.2. Culture of organisms

3.7.2.1. Homogenization, decontamination and concentration by N-Acetyl-L-Cysteine-Sodium hydroxide (NALC-NaOH) method

The specimens submitted for mycobacterial culture includes expectorated or induced sputum, gastric lavage, transtracheal aspiration, bronchoscopic aspiration, urine, fecal specimen, tissue and body fluid specimens, blood, wound or skin lesion aspirate, lymph node aspirates etc. Tissue or body fluids like Cerebrospinal fluid, pleural fluid, joint fluid; bone marrow aspirates etc. collected aseptically usually do not require digestion and decontamination and can be cultured directly. However majority of specimens submitted for mycobacterial culture consist of organic debris like mucin, tissue, serum and other proteinaceous material that is contaminated with other bacteria which can rapidly outgrow mycobacteria in culture so although all digestion and decontamination methods have their own limitations, those which can release mycobacteria from mucin or cells, maximize the survival and detection of mycobacteria and maximize the elimination of contaminating organisms are used.

Sputum obtained from patients consists of a complex organic matrix contaminated with a variety of microorganisms that can rapidly outgrow the mycobacteria (Nolte & Metchock, 1995). Mucin

may trap mycobacterial cells and protect contaminating microorganisms from the action of decontaminating agent. So the sputum should be digested and decontaminated with suitable agents that liquefy sputum and release mycobacterial cells and eliminate contaminating agents by least hampering mycobacterial cells.

NaOH is a commonly used decontaminant that is also mucolytic, but because of its potential toxicity to mycobacteria, it should be used at the lowest concentration that effectively digests and decontaminates the specimen. NALC is a mucolytic agent which on addition reduces the concentration of NaOH required and also shortens the time required for decontamination, thus aiding the optimal recovery of mycobacteria (Forbes et al., 2007). The centrifugation after processing with NALC-NaOH concentrates the mycobacteria in the sediment.

3.7.2.2. Culture

The ideal medium for mycobacterial culture should support the most rapid and abundant growth, allows for the study of colony morphology and pigment production, inhibits the growth of contaminants, and is economical. The media available for mycobacterial culture may be agar based solid media like Middlebrook 7H10, Middlebrook 7H11 agar media; egg based solid media like Lowenstein-Jenson media, Ogawa media; or liquid media like Middlebrook 7H9 broth, Dubos broth, BACTEC 12B medium (Forbes et al., 2007).

The sloped L-J medium is inoculated with processed and concentrated specimen by streaking, cap replaced, and incubated at 35ºC to 37ºC, preferably in an atmosphere of 5 to 10% Carbon dioxide and high humidity. The tubes are observed after 24 to 48 hours for contamination, in a week for rapid growers, and then weekly upto 8 weeks for growth of *M. tuberculosis* complex or slow growing NTM. *M. tuberculosis* gives rough, buff and tough colonies on LJ whereas NTM may give rough to smooth, colourless to yellow to pink pigmented, tough or soft depending on species. If growth occurs within 8 weeks it is reported as shown in Appendix VI; if fungal growth occurs it is regarded as contaminant; and if no growth occurs it is regarded as negative for mycobacterial growth.

3.7.3. Approach for identification of pathogen

The organism growing on solid or liquid media is first acid fast stained to confirm whether it is acid fast organism or not. The approach of identification of the mycobacteria upto species level may include observation of growth characteristics, biochemical tests cell wall lipid analysis or molecular methods.

3.7.3.1. Growth characteristics observation

The preliminary identification of mycobacterial isolates depends upon their growth rate, colonial morphology, colonial texture, pigmentation and in some instances the permissive incubation temperature for their growth. The growth rate is the amount of time required for mature colonies to be visible on solid media without magnification (Nolte & Metchock, 1995). Rapid growers produce colonies within seven days on subculture, so can be separated from slow growing NTM and *M. tuberculosis* complex. But the dilution used for subculture is critical which if more concentrated make even slow growers to be observed within seven days, so *M. flavescens* can be used in quality control to prevent its exhibition as false positive rapid grower(Forbes et al., 2002).

The rough or smooth texture, tough or soft consistency, the opacity of the colony, it's size, pigmentation, and emulsification of colonies in suspension may provide preliminary insight to an experienced technician in the identification of the species. The colonial features provide presumptive identification of the organisms and suggest the biochemical tests or nucleic acid probes to be used for definitive identification (Nolte & Metchock, 1995).

Slow growing NTM can be categorized into three groups, viz. photochromogens, scotochromogens and non photochromogens, on the basis of pigmentation. To achieve optimum photochromogenicity, colonies should be young, actively metabolizing, isolated and well aerated (Wayne, 1964). Many mycobacterial species synthesize carotenoids pigments which are yellow to red in colour. Photochromogens produce non pigmented colonies when grown in the dark and pigmented colonies only after exposure to light and reincubation. Scotochromogens produce

deep yellow to orange pigmented colonies irrespective of whether grown in light or dark; however some strains may have an increased pigment production upon continuous exposure to light. Nonphotochromogens are nonpigmented whether grown in the dark or light or have only a pale yellow, buff, or tan pigment that does not intensify after light exposure (Nolte & Metchock, 1995). *M. tuberculosis* complex can be separated from nonphotochromogenic slow growing NTM on the basis of colony morphology, growth on PNB media and biochemical tests.

3.7.3.2 Biochemical tests

Once preliminarily sub grouped based on its growth characteristics, an organism must be definitively identified to species or complex level for effective chemotherapy. Conventional biochemical tests are still the main tools in this approach in poor laboratory settings, whereas sophisticated laboratories are using molecular methods as well.

3.7.3.2.1. Niacin test

Niacin (nicotinic acid) functions as a precursor in the biosynthesis of coenzymes NAD and NADP which play important role in oxidation-reduction reactions in mycobacterial metabolism. Although all species produce niacin, *M. tuberculosis* accumulates it in largest amount in the growth medium because of a blocked metabolic pathway for conversion of free niacin to nicotinic acid mononucleotide (Nolte & Metchock, 1995; Forbes et al., 2002). The positive niacin test of slow growing mycobacteria possessing rough surfaced, buff-coloured and tough colonies gives preliminary identification of *M. tuberculosis*; however the test is not itself sufficient for definitive confirmation. Niacin negative *M. tuberculosis* isolates are rare but some strains of *M. simiae*, *M. bovis* BCG, and other mycobacteria, although infrequently encountered, also accumulate niacin (Nolte & Metchock, 1995). For reliable results, the niacin test should be performed only from cultures on L-J that are at least 3 weeks old and show at least 50 colonies; otherwise enough niacin may not be present for detection (Forbes et al, 2007).

3.7.3.2.2. Nitrate reduction test

Certain mycobacterial species utilize nitrate as terminal electron accepter so nitrate gets reduced. The ability of acid-fast bacilli to reduce nitrate is influenced by age of the colonies, temperature, pH, and enzyme inhibitors (Forbes et al., 2002). Nitrate reduction test is valuable for the identification of some mycobacteria that possess similar colony morphology, growth rate, and pigmentation characteristics (Nolte & Metchock, 1995). This test is useful for the identification of *M. tuberculosis*, *M. kansasii*, *M. szulgai* and *M. fortuitum* which are nitrate reducers. Although rapid-growers can be tested within two weeks, slow-growers should be tested after 3 to 4 weeks of luxuriant growth (Forbes et al., 2007).

3.7.3.2.3. Heat labile (68ºC) Catalase test

Most species of mycobacteria, except for certain strains of *M. tuberculosis* complex (some Isoniazid resistant strains), some non pathogenic isoniazid resistant strains of *M. kansasii* and *M. gastri*, produce the intracellular enzyme Catalase, which splits hydrogen peroxide into water and oxygen (Forbes et al., 2002). Mycobacterial species can be distinguished by quantitative difference in Catalase activity demonstrated by intact cells in the semiquantitative catalase test and by the differences in heat stability detected by the 68ºC catalase test (Nolte & Metchock, 1995). Heat stable catalase test is assessed by the ability of the catalase enzyme to remain active after heating the mycobacterial suspension in pH 7 buffer to 68ºC for 20 minutes, in which the catalase of *M. tuberculosis*, *M. bovis*, *M. gastri* and *M. haemophilum* gets inactivated but that of some photochromogens, scotochromogens, nonphotochromogens and rapid growers still remain active and helps in identification of species (Forbes et al., 2007).

3.7.3.2.4. Growth on PNB containing media

Human or bovine types of tubercle bacilli can be differentiated from all other mycobacteria with their inability to grow in the LJ medium containing 500µg/ml of para-nitro-benzoic acid. Occasionally human strain may give some faint growth in this medium. The results of growth on

PNB containing media and niacin test helps in typing human and bovine tubercle bacilli strains (NTI , 1998).

3.7.4. BACTEC TB-460 radiometric culture systems

BACTEC TB-460 (Becton Dickinson, Sparks, MD) is automatic system using radiometric instrumentation and uses vials containing modified Middlebrook 7H9 broth medium with one of its components, palmitic acid, is radio-labeled with C-14 for mycobacterial culture. Contamination is controlled by addition of a mixture of polymixin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin in a poly-oxyethylene solution (Watt et al., 2006). The vials containing the medium remain sealed through the whole culture process and the specimen after decontamination is inoculated by puncturing the rubber septum with a needle. The vials which are held in an external incubator must be loaded into the instrument for reading. When viable mycobacteria are present in the culture vial, the radio-labeled palmitic acid is metabolized and radioactive CO_2 is liberated into the gaseous phase. The gaseous phase is aspirated, replaced by air with 5% CO_2, and analyzed by a β-counter to quantify the eventual presence of radio-labeled CO_2. The reading is usually performed twice a week during the first 15 days of incubation and weekly thereafter until the 42nd day (Forbes et al., 2002).

3.7.5. Polymerase chain reaction based nucleic acid amplification method

First proposed genomic target for diagnostic PCR was the insertion element IS6110 which being present in multiple copies (from 4 to 20 in more than 95% of *M. tuberculosis* strains) have potential for enhanced sensitivity. Other successfully used DNA regions include the 65 kDa heat-shock protein gene, the gene encoding the 126 kDa fusion proteins, and the gene encoding the β-subunit of RNA polymerase; all of them are present in single copies in *M. tuberculosis* complex genomes (Palomino et al., 2007).

3.7.6. Tuberculin skin test (TST)

TST has been used to identify patients with active tuberculosis, to measure the prevalence of infection in a community, and to select susceptible or high risk individuals for BCG vaccination

(Palomino, 2007). Tuberculin is the test antigen and is of two types, viz. old tuberculin (OT) and the purified protein derivative (PPD), the later is the purer preparation, gives fewer non-specific reactions and is easier to standardize (Park, 2007). These antigens are standardized in terms of their biological reactivity as "tuberculin units"(TU). One TU is equal to 0.01ml of OT or 0.00002 mg PPD and standard PPD maintained by WHO contains arbitrarily 50,000 TU per milligrams (Park, 2007). Tuberculin has also been prepared from atypical mycobacteria, like PPD-B from Battey mycobacteria, PPD-Y from *M. kansasii*, scrofulin from *M. scrofulaceum*, etc. , which are used in epidemiological surveillance and many of them cross-react (Park, 2007). The dosage of PPD are available in first strength or 1 TU, intermediate strength or 5 TU and second strength or 250 TU; and nearly all infected persons react to 1 or 5 TU and stronger doses elicit a higher proportion of false positive (Park, 2007). In tuberculin skin test 0.1 ml of 5 TU PPD is injected in the fore arm and on examination after 48-72 hours, the positive reaction is indicated by the presence of erythema and induration >10 mm in size. All persons with prior infection with tubercle bacilli will mount an immune response to bacilli protein, but the protein antigen used in PPD is shared by BCG vaccine and environmental mycobacteria so often false positive result, almost one third is estimated, is obtained if used in TB diagnosis alone (Curley, 2003; Palomino, 2007). The sensitivity of the skin test is estimated to be around just 70% in known active TB cases, which decreases to as low as 30% in immunocompromised people (Palomino, 2007). If people are regularly screened for TB infection using skin test, they start to become immunized to PPD by its repeated administration, which is called boosting and results in a false positive reaction in the skin test (Palomino, 2007).

3.8. Drug susceptibility testing of mycobacteria

Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug (Mitchison, 1962). Drug susceptibility tests are done to find out whether the organisms isolated in culture are sensitive or resistant to the drugs used in the treatment of the patient. Drug susceptibility tests of mycobacteria can be done either direct or indirect methods. The direct method uses a smear positive concentrate containing more than 50 acid fast bacilli per 100 oil immersion fields as the inoculums; whereas the indirect method uses

a culture as the inoculums source (Forbes et al., 2002). Direct method is faster than indirect method because no time is lost to get primary culture but contamination of drug slopes may be comparatively higher (NTI, 1998). In indirect method the nature of the culture and its appearance are well known, size of inoculums can be easily controlled and contamination rate is less, so is most commonly used.

Drug susceptibility of mycobacteria is done by various methods. In Absolute concentration method, for the test of each drug, a standardized inoculum is inoculated to drug free control media and media containing several appropriate graded drug concentration; and the resistance is expressed as the lowest concentration of drug that inhibits all or almost all of the growth, which is the minimum inhibitory concentration (MIC) (Forbes et al., 2007).

In Resistance ratio method, the resistance of test organism is compared with that of standard laboratory strain *M. tuberculosis* $H_{37}Rv$. Both strains are tested in parallel by inoculating a standard inoculum to media containing twofold serial dilutions of the drug; and resistance is expressed as the ratio of MIC of the test strain and standard laboratory sensitive strain $H_{37}Rv$ for each drug and same set of tests (Forbes et al., 2007; Canetti et al., 1969).

All strains of tubercle bacilli contain some bacilli that are resistant to anti-tuberculosis drugs; however in resistant strains, the proportion of such bacilli is considerably higher than in sensitive strains. The proportion of resistant bacilli present in a strain is calculated by proportion method. Two appropriate bacillary dilutions are made, such that, upon inoculation of each on drug containing and drug free medium, numerable colonies are obtained on both media; then the ratio of the number of colonies obtained on the drug-containing media to that of drug-free media indicates the proportion of resistant bacilli present in the strain (Canetti et al., 1969). If growth at the critical concentration of a drug is 1%, the isolate is considered clinically sensitive; and if >1% it is considered clinically resistant (Forbes et al., 2007).

3.9. Treatment of mycobacterial diseases

For the treatment of TB, the available anti TB drugs are Isoniazid, Rifampicin, Pyrazinamide, Streptomycin, Ethambutol, Thiacetazone, Kanamycin, Capreomycin, PAS, Ethionamide,

Prothionamide and Cycloserine. The newer and experimental drugs for MDR-TB are Ciprofloxacin, Ofloxacin, Pefloxacin, Lomefloxacin, Sparfloxacin, Roxithromycin, Clarithromycin, Azithromycin and Amikacin (Jain et al., 2001). Nepal NTP adopted DOTS strategy in 1996 and nationwide coverage was achieved in 2001. NTP offers fixed dose combination (FDC) tablets of HRZE, HRE and HR combinations for following reasons:

- To prevent monotherapy.
- To reduce emergence of drug resistant TB.
- To simplify treatment and minimize prescription error.
- To increase patient and treatment provider compliance.
- To simplify drug stock management, shipping and distribution.

Table 2: NTP TB treatment category, regimen and duration of treatment (NTC, 2011)

Category	Regimen and duration in months(m)	Type of patients
I	2m (HRZE) + 4m (HR)	• New sputum smear positive • Seriously ill* new sputum smear negative • Seriously ill* new extra-pulmonary
II	2mS (HRZE) + 1m (HRZE) + 5m (HRE)	Retreatment TB cases including failures, relapse and return after default.

*includes new sputum smear negative TB patients with extensive parenchymal involvement, severe form of extra-pulmonary TB. Seriously ill also includes any patients, pulmonary or extra-pulmonary who is HIV positive.

Pulmonary mycobacteriosis is also treated with anti-TB or other drugs on basis of identification of species and drug susceptibility testing and hospitals have their own strategy for the treatment of NTM infection and disease.

CHAPTER IV

4. MATERIALS AND METHODS

4.1. Materials

A complete list of bacteriological media, chemicals, reagents, equipments, instruments, glass wares and other miscellaneous materials that were required is given in Appendices I-III.

4.2. Methodology

The study was conducted at National Tuberculosis Center (NTC), Thimi, Bhaktapur from September 2010 to August 2011. The study was of cross-sectional type. The new patients suspected for pulmonary tuberculosis were included in the study but already diagnosed cases, treatment failure cases and follow up cases were not included in the study. Both male and female of all age groups, satisfying Bartlett's inclusion criteria given in Appendix VI for pulmonary specimen culture, were included in the study.

4.2.1. Sample collection

4.2.1.1. Consent

The written consent was taken from most of the participants of the study and verbal consent was taken from some illiterate or unwilling patients to sign in the document not related to the mainstream diagnostic and treatment programme of the hospital, after brief description of objectives and rationale of the study. The participants were completely free whether to take part or not in the study.

4.2.1.2. Questionnaire

A brief questionnaire was filled up by the investigator after verbal interview with the participants of the study.

4.2.1.3. Specimen

Sputum was the specimen that was collected from the study population. Three sputum samples were collected from each individual as:

i. Sputum taken out from the patient on the spot at NTC when s/he first reached the center for diagnosis.
ii. The first early morning sputum taken out at home of the patient on the following day.
iii. Sputum taken out again on the spot at NTC when s/he delivered the early morning sputum on the second day.

The specimens were collected in a wide mouthed, sterile, transparent and leak proof plastic containers with lid. Patients were instructed to rinse their mouth with water and cough forcibly

facing the wall away from other people by keeping hands on hips and spit sputum carefully into container and close lid tightly.

4.2.2. Macroscopic observation of specimen

The sputum was observed macroscopically for differentiation of sputum into purulent, mucopurulent, mucoid, mucosalivary of haemorrhagic.

Table 3: Macroscopic observation of sputum (Cheesbrough, 2002)

S.N.	Type	Observation
1	Purulent	Green looking, mostly pus
2	Mucopurulent	Green looking with pus and mucus
3	Mucoid	Mostly mucus
4	Mucosalivary	Mucus with small amount of saliva
5	Haemorrhagic	Presence of blood

4.2.3. Processing of specimen

The sputum sample was processed within two hours for microscopy and culture, if not possible, it was stored at 4ºC for future processing within a week.

4.2.3.1 Ziehl -Neelsen staining procedure

A new, clean, unscratched, grease free slide was taken and labeled at one side with the relevant patient number with diamond pencil. Inside BSC, an appropriate portion of the sputum; blood specks, mucus if available; was transferred from sputum container to the slide with applicator stick and smeared to make smear of 3×2 cm in middle portion of the side without touching edges, and kept in the metal rack for air drying. Then the slide was holded with blunt forcep and passed over the flame 3-4 times, cooled and placed over staining tray. The smear was covered by Carbol fuchsin and heated from downside with burning cotton soaked in spirit till steam came and slides were not allowed to dry and left for 5 minutes. The slide was washed with distilled water, the water drained and then decolourized by applying 20% Sulphuric acid for 5 minutes. More acid may be added and time may be increased to make the smear colourless. Then the slide was washed with distilled water, excess water drained, and then covered with 0.1% Methylene blue for 30 seconds. The slide was then washed with distilled water, dried, observed under oil immersion objective and then reported as shown in Appendix VI.

4.2.3.2. Fluorochrome staining procedure

A new, clean, unscratched, grease free slide was taken and labeled at one side with the relevant patient number with diamond pencil. Inside BSC, an appropriate portion of the sputum; blood specks, mucus if available; was transferred from sputum container to the slide with applicator stick and smeared to make smear of 3 × 2 cm in middle portion of the side without touching

edges, and kept in the metal rack for air drying. Then the slide was holded with blunt forcep and passed over the flame 3-4 times, cooled and placed over staining tray. The smear was covered by Auramine phenol solution and left for 15 minutes. The slide was washed with distilled water, the water drained and then decolourized by applying 20% Sulphuric acid for 5 minutes. Then the slide was washed with distilled water, excess water drained, and then covered with 0.1% Methylene blue for 30 seconds. The slide was then washed with distilled water, dried, observed under 20 × objective and then reported as shown in Appendix VI.

4.2.3.3. Culture of specimen

The sputum samples were cultured on duplicate LJ media after digestion and decontamination of sputum.

- **Digestion and decontamination of sputum by NALC-NaOH method**

Working within BSC and wearing protective clothing, gloves and mask, a maximum of 10 ml of sputum was transferred to a sterile, disposable, plastic, 50ml conical centrifuge tube with volume indicator and leak proof and aerosol free plastic screw cap. Equal volume of freshly prepared NALC-NaOH digestant was added without touching lip of specimen container and the cap was tightened. The specimen was vortexed for approximately 15-30 seconds. The tube was inverted to check the homogeneity of specimen. If clumps remain, the specimen was vortexed intermittently while the rest of the specimens were being vortexed. An extra pinch of NALC crystals may be necessary to liquefy mucoid sputa. A 15 minute timer was started after vortexing first specimen and other specimens were digested noting the time that entire run will take. The digestant may remain on the specimens for a maximum exposure of 20 minutes. After 15 minutes of digestion, enough phosphate buffer was added to reach within 1cm of the top, then the cap was closed tightly and the tube was inverted to mix the solutions and stop the digestion process. Addition of phosphate buffer also reduces the specific gravity of the specimen, aiding sedimentation of the bacilli during centrifugation. All tubes were centrifuged at 3000×g for 20 minutes, using aerosol free sealed centrifuge cups. The supernatant was poured off carefully into a splash proof container containing 5% Phenol without touching lips of container. To ensure that the specimen did not run down the outside of the tube after pouring, the lip of the tube were wiped with an amphyl- or phenol soaked gauze to absorb drips. If sediment began to slip during pouring the decantation was stopped and sterile capillary pipette was used to remove the supernatant without losing the sediment. The sediment was resuspended in 1 to 2 ml of phosphate buffer with pH 6.8

- **Culture of the sputum**

A loopful (3 mm internal diameter Nichrome loop) of sediment after digestion and decontamination was inoculated to each of duplicate LJ media tubes and incubated at 37 ºC. The tubes were observed after 24-48 hours to discard contaminated tubes. Then the tubes were observed weekly upto 8 weeks to report the culture as in Appendix VI, and the negative and contaminated tubes were discarded.

4.2.3.4. Determination of pigment production and growth rate

The suspension of bacterial colonies was made in distilled water and turbidity adjusted to that of a Mc Farland 1 standard. Then 0.1 ml of suspension was inoculated to each of two tubes of LJ agar slopes; one was completely wrapped by Aluminium foil to block all light and next was left for observation; and then incubated at 37 ºC. The cultures were examined upto 7 days to detect rapid grower. The rapid growers may give non-chromogenic to chromogenic with yellow to pink colonies and from smooth to rough colonies. Then the cultures were observed weekly on unwrapped tubes to detect slow growers and when mature colonies formed, both tubes were observed to detect scotochromogens and colony characteristics noted; and if no pigmentation in colonies on any tubes, one foil wrapped tube was exposed to tungsten lamp for 2 to 5 hours with cap loosened, then the tube was wrapped again and returned to incubator with cap left loose and examined after 24 and 48 hours to detect whether pigmentation occured in previously non-pigmented colonies to detect photochromogens and colony characters noted, the non pigmented may belong to *M. tuberculosis* complex or nonphotochromogenic NTM and their colony characters noted.

4.2.3.5. Identification biochemical tests

- **Niacin test by Modified Runyon method**

The 3 to 4 weeks old subcultures having approximately 50-100 colonies were taken along with *M. tuberculosis* $H_{37}Rv$ and plain LJ as positive and negative control respectively. The slopes were punctured with a sterile inoculation wire to extract niacin from the medium. Then 1ml of sterile distilled water was added to each slope and kept in a horizontal position for 30 minutes and then autoclaved for 15 minutes at 121 ºC. Then 0.25 ml of autoclaved extract was taken in a test tube and equal amount of 4% aniline in ethanol and again, equal amount of 10% aqueous Cyanogen bromide were added to it, and observed for appearance of yellow colour. At last, 2-3 ml of 4% NaOH was added to each tube and discarded.

- **Nitrate reduction test**

About 0.2 ml of sterile distilled water was taken in a 16-×125- mm screw capped tube and two very large clumps of growth from a 4-week test culture on LJ agar was emulsified to make milky suspension. *M. tuberculosis* $H_{37}Rv$ culture and reagent blank were taken as positive and negative control. Then 2 ml of Nitrate substrate broth was added to the suspension and capped tightly. It was gently shook and incubated for 2 hours in a 35˚C water bath in upright position. After two hours of incubation the following reagents were added in order:

a. 1 drop of dilute Hydrochloric acid.
b. 2 drops of 0.02% Sulphanilamide
c. 2 drops of 0.01% N-Naphthylethylene-diamine

Then the tube was examined immediately for formation of pink to red colour and the reading was taken as:

Colour observation	**Reading**
Faint pink	+/-
Clear pink	1+
Deep pink	2+
Red	3+
Deep red	4+
Purple red	5+

Interpretation

3+ to 5+: positive

No colour formation, 1+ to 2+: negative

True negatives were confirmed by adding a small amount of zinc powder to all negatives. If there was $NaNO_3$ in the suspension, after addition of zinc it turned to pink colour and it was true negative. If there is no colour formation after addition of zinc it means the reaction went further than nitrate reduction and it was a positive reaction.

- **Heat labile Catalase test**

About 0.5ml of 0.067 M phosphate buffer, pH 7 was taken in screw capped tube and several loops full of colonies from test cultures were emulsified. The tubes were placed in a previously heated water bath at 68°C for 20 minutes. The time and temperature were critical. Then the tubes were removed and after cooling to room temperature, 0.5 ml of freshly prepared Tween-peroxide was added to each tube and caps were loosely fitted and tubes rested calmly. The tubes were observed for bubble formation without shaking and negative tubes were held for 20 minutes before discarding.

- **Growth in the presence of 0.5mg/ml p-Nitrobenzoic acid (PNB)**

The bacterial suspension was made in 0.2ml of distilled water by emulsifying several loops full of colonies from test culture and diluted to make suspension with turbidity similar to Mc Farland's 1 standard. LJ media slope with 0.5 mg/ml PNB was inoculated with one loop full (3 mm internal diameter) of the suspension and incubated at 37 °C for 1 to 1.5 months. After incubation the media was observed for growth and result interpreted.

4.2.3.6. Antibiotic susceptibility testing by proportion method

- **Preparation of drug containing media**

The drug containing media were prepared with different concentration of anti tubercular drugs as shown in Appendix II

- **Preparation of bacillary suspension**

Inside BSC, 0.1ml of sterile distilled water was taken in a screw capped tube containing sterile glass beads and one loopful (3mm diameter loop) of growth of test culture was transferred to it and emulsified. The tube was vortexed for few minutes to give smooth suspension. Leaving sediment the suspension was decanted to another sterile tube and its turbidity was adjusted to that of Mc Farland No.1 standard to make 1 mg/ml bacillary suspension. This was 'neat suspension'. The 'neat suspension' of standard laboratory strain *M. tuberculosis* $H_{37}Rv$ was also prepared for quality control.

- **Dilution of neat suspension for inoculation**

0.01 ml of neat suspension was mixed to 1 ml of sterile distilled water to make 10^{-2} dilution and further diluted to make 10^{-4} suspension.

- **Inoculation and incubation**

From 10^{-2} dilution, four LJ drug containing media tubes containing anti-tubercular drugs in critical concentration and one control plain LJ tube were inoculated. From 10^{-4} dilution, four LJ drug containing media tubes containing anti-tubercular drugs in critical concentration and two controls plain LJ tubes were inoculated. From 10^{-3} of 'neat suspension' of *M. tuberculosis* $H_{37}Rv$, various graded drug containing media were inoculated for quality control. All inoculated tubes were incubated at 37 ºC.

- **Interpretation of results**

For each dilution of test culture, the number of colonies in drug containing media and drug free LJ media were counted.

Table 4: Critical concentrations of primary anti-tubercular drugs used in the study

S.N.	Drug used	Concentration(µg/ml)
1	Isoniazid	0.20
2	Rifampicin	40
3	Streptomycin	4
4	Ethambutol	2

The result was interpreted as follows:
Sensitive (S): If growth at the critical concentration of the drug is less than or equal to that of 1% control media without the drug.
Resistant (R): If growth at the critical concentration of the drug is greater than that of 1% control media without the drug.

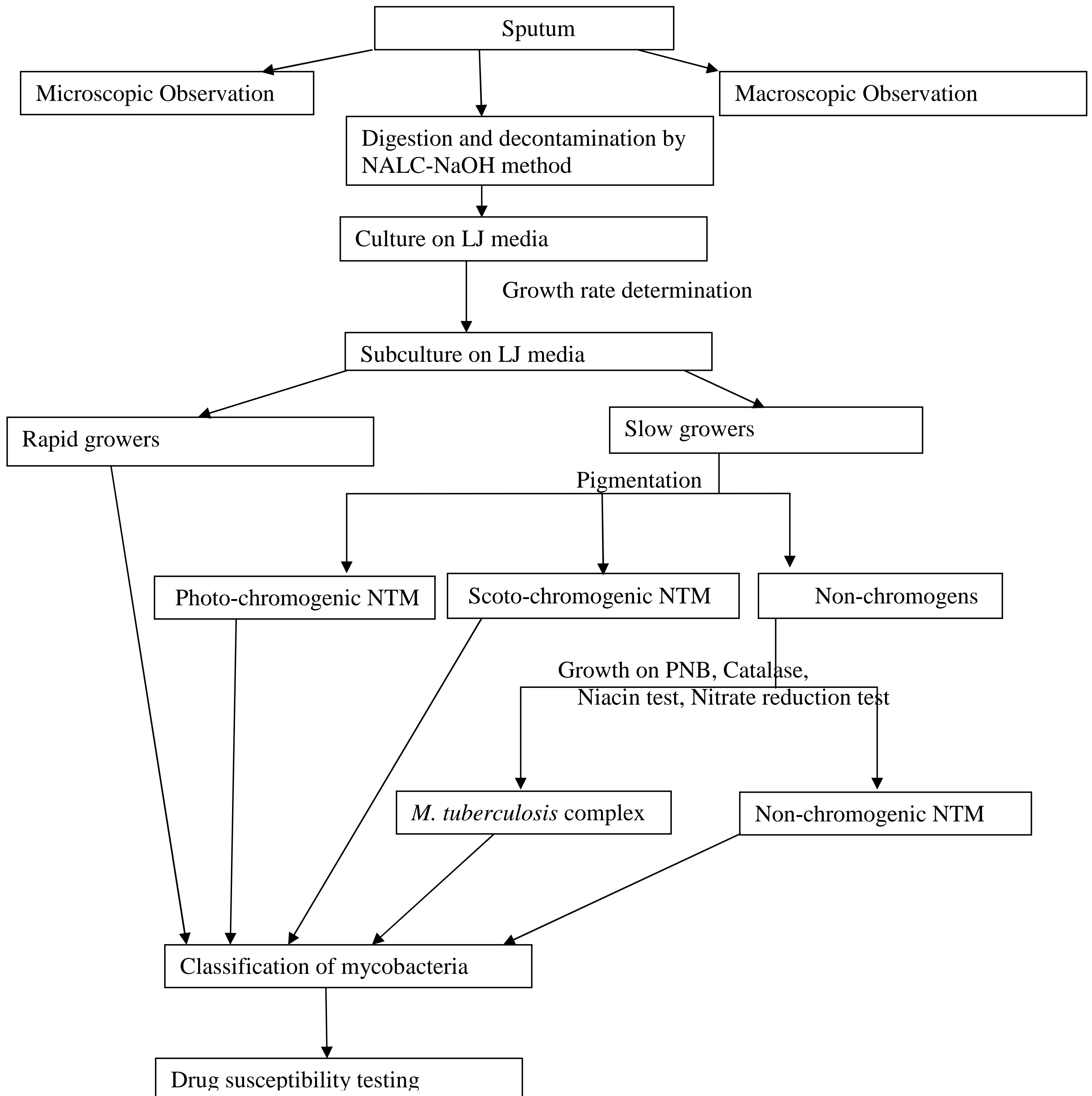

Figure 1: Flowchart of Methodology

CHAPTER V

5. RESULTS

This study was carried out at NTC during September 2010 to August 2011 in order to isolate, classify and determination of antibiotic susceptibility of *Mycobacterium* species associated with TB and pulmonary mycobacteriosis, present in the sputum samples of suspected new PTB patients. A total of 200 patients were selected from 1500 patients on basis of the satisfaction of their sputum sample for culture according to Barlett's inclusion criteria which rejects more sputum samples but rejects less pathogens which is quite important for expensive mycobacterial culture (Wong et al., 1982).

All selected patients were interviewed to obtain their demographic characteristics as well as their habit of smoking, alcoholism, family history of TB and BCG vaccination. Three sputum samples were collected from each patient and cultured. *Mycobacterium* species were classified on basis of their growth rate, pigmentation of colonies and biochemical tests of Culture positive isolates and all culture positive isolates were subjected to antibiotic susceptibility test.

129 sputum samples were Culture positive among 200 patients sputum samples selected by Barlett's pulmonary specimen culture criteria. Among 129 culture positive 84.60% (n=113) had TB and 12.40% (n=16) had pulmonary mycobacteriosis. *M. tuberculosis* was responsible for TB cases and 8% (n=9) of isolated *M. tuberculosis* strains were found as MDR strains resistant to at least INH and RMP. Nonphotochromogens were responsible for 81.25% (n=13), Scotochromogens for 12.50% (n=2) and Rapid grower *M. vaccae* was responsible for 6.25% (n=1) of total pulmonary mycobacteriosis cases. Among total NTM, 6.25% (n=1) was resistant to INH and STR only and rest 93.75% (n=15) were resistant to all four anti-tubercular drugs INH, RMP, STR and EMB.

The association of mycobacterial infection with family history of TB and Animal husbandry was statistically significant, but former has positive association and later has negative association. The association of Smoking, Alcoholism and BCG vaccination with mycobacterial infection was not statistically significant.

5.1. Distribution of total study cases (N=200) by age and sex

Among the studied 200 cases, 71 % (n=142) were male and 29% (n=29) were female with highest number of cases in age group 20-30 (27.5%), followed by 30-40 (23.5%).

Table 5: Age and Sex distribution of total cases

Age in years	Male		Female		Total	
	No.	%	No.	%	No.	%
10-20	8	4	5	2.5	13	6.5
20-30	32	16	23	11.5	55	27.5
30-40	35	17.5	12	6	47	23.5
40-50	18	9	10	5	28	14
50-60	25	12.5	5	2.5	30	15
60-70	18	9	1	0.5	19	9.5
70-80	6	3	2	1	8	4
Total	142	71	58	29	200	100

5.2. Distribution of *Mycobacterium* positive cases in sputum with Ziehl-Neelsen staining, Fluorescent staining and Culture

Among 200 patients selected by Bartlett's inclusion criteria, 138 patients, 71.01 % (n=98) male and 28.99% (n=40) female, were positive for *Mycobacterium* either singly or multiply with Ziehl-Neelsen staining, Fluorescent staining or Culture,. Among total 200 sputum specimens, 64.5% (n=129) were Culture positive, 31.5% (n=63) were Culture negative and 4% (n=8) were contaminated.

Table 6: Comparison of Ziehl-Neelsen staining, Fluorescent staining and Culture of total *Mycobacterium* positive cases selected by Bartlett's inclusion criteria

Staining/Culture	Male		Female		Total	
	No.	%	No.	%	No.	%
Ziehl- Neelsen	87	63.04	38	27.54	125	90.58
Fluorescent	91	65.94	37	26.81	128	92.75
Culture	92	66.67	37	26.81	129	93.48
Total of positive	98	71.01	40	28.99	138	100

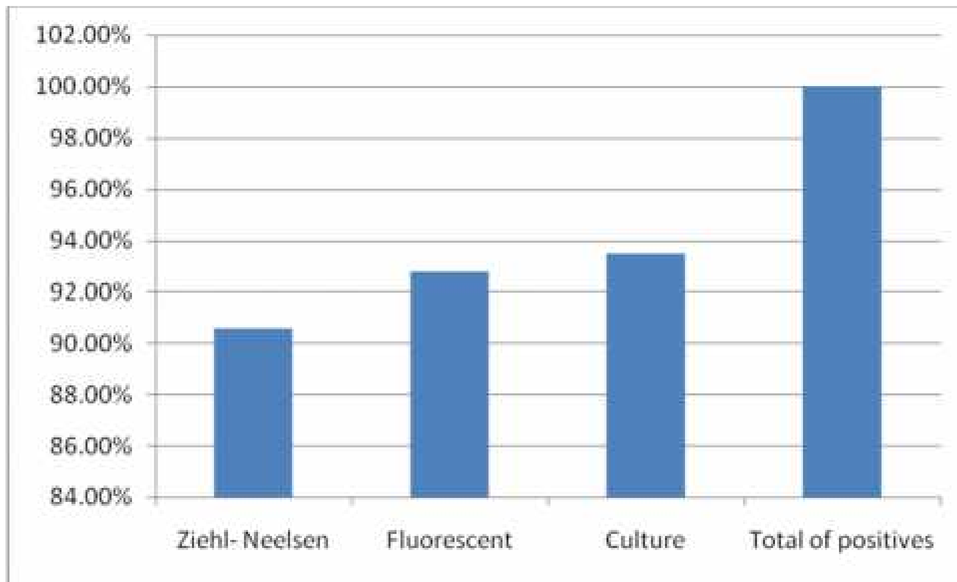

Figure 2: Comparison of Ziehl-Neelson staining, Fluorescent staining and Culture of *Mycobacterium* positive cases

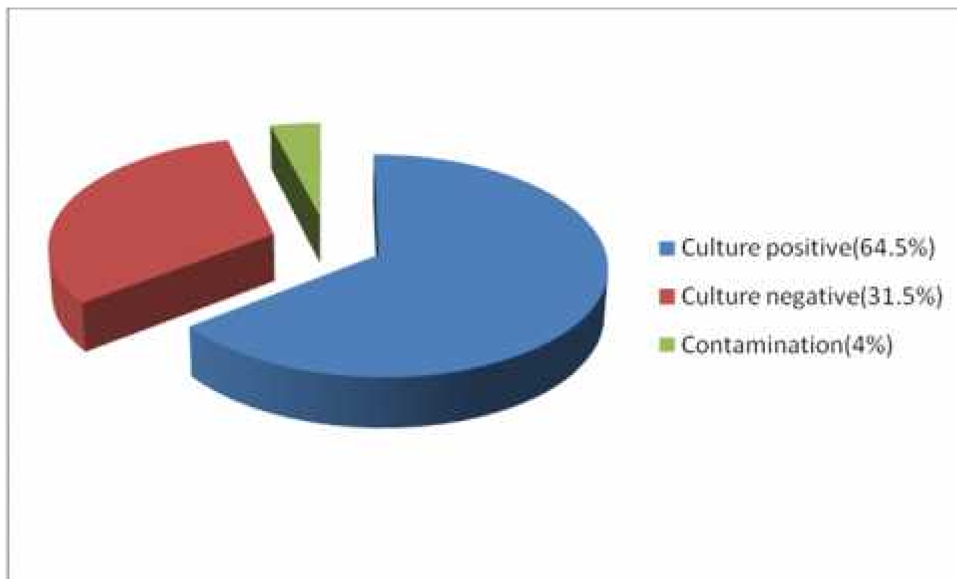

Figure 3: Distribution of Culture positive, negative and contamination among total specimens

The sensitivity of ZN staining, Fluorescent staining and culture with respect to total positive either singly or multiply by these methods were in increasing order 90.58%, 92.75% and 93.48% respectively.

Since, culture is considered as gold standard; ZN and fluorescent microscopy were compared to it for the comparision.

Table 7: Comparison of ZN microscopy with Culture

ZN microscopy	Culture		Sensitivity %	Specificity %	Predictive value of +ve test%	Predictive value of -ve test %	False negatives %	False positive %
	+ve	-ve						
Positive	119	6	90.84	91.18	95.20	83.78	9.16	8.82
Negative	12	62						

Fluorescent microscopy was found to be more sensitive and specific than ZN microscopy.

Table 8: Comparison of Fluorescent microscopy with Culture

Fluorescent microscopy	Culture		Sensitivity %	Specificity %	Predictive value of +ve test%	Predictive value of -ve test %	False negatives %	False positive %
	+ve	-ve						
Positive	122	6	94.57	91.54	95.31	90.28	5.43	8.45
Negative	7	65						

5.3. Distribution of PTB and pulmonary mycobacteriosis among culture positive cases

Among 129 Culture positive cases, 87.60% (n=113) person had TB of which 61.24% (n=79) were male and 26.36% (n=34) were female. The causative organism of TB was *M. tuberculosis.*

Another 12.40% (n=16) of total 129 Culture positive cases had pulmonary mycobacteriosis other than TB. Among them 10.08% (n=13) were male and 2.32% (n=3) were female. The causative organism was either Scotochromogens or Non photochromogens or Rapid grower NTM.

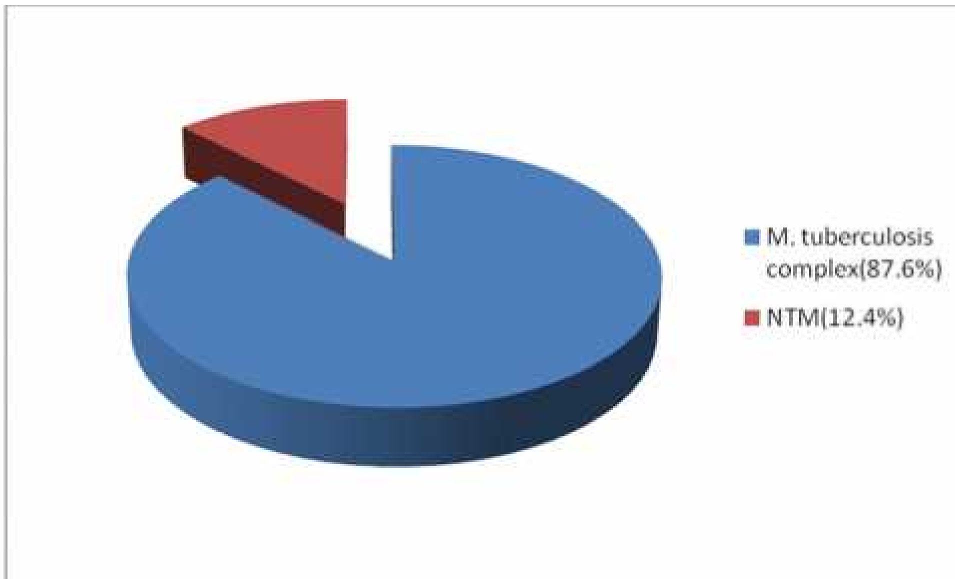

Figure 4: Distribution of *M. tuberculosis* complex and Non- tuberculous mycobacteria in culture positive cases

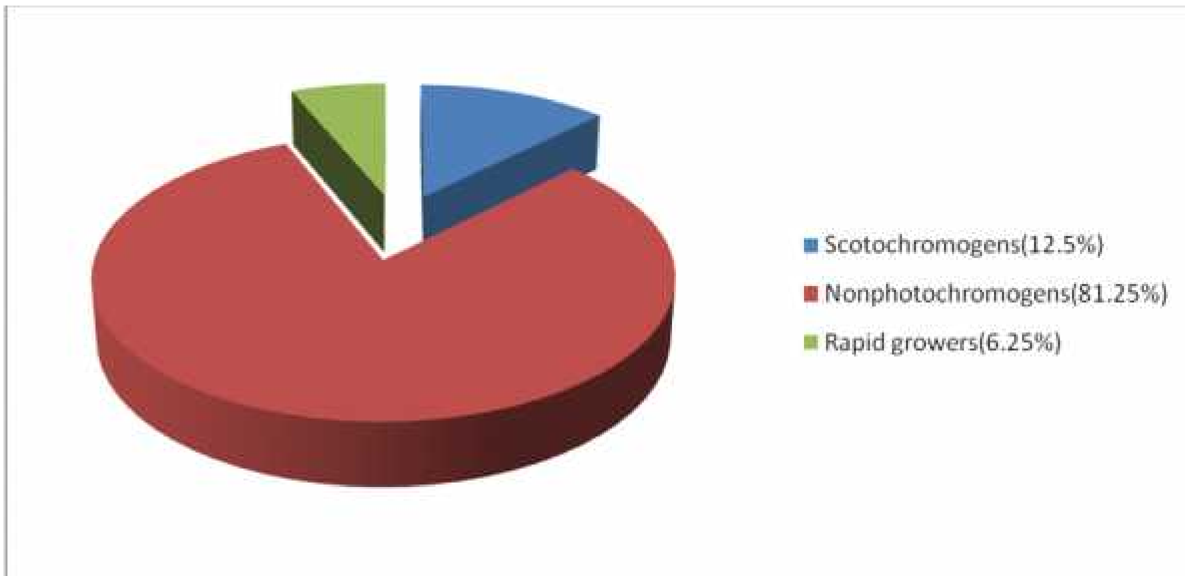

Figure 5: Distribution of NTM in culture positive cases

Table 9: Distribution of *M. tuberculosis* complex and NTM in Culture positive cases

Infectious agent	Male		Female		Total	
	No.	%	No.	%	No.	%
M. tuberculosis complex	79	61.24	34	26.36	113	87.60
NTM	13	10.08	3	2.32	16	12.40
Total	92	71.32	37	28.68	129	100.00

5.4. Distribution of pulmonary mycobacteriosis on basis of NTM classification

Among 16 pulmonary mycobacteriosis patients, 81.25% (n=13) were infected by Non-photochromogens among them 68.75% (n=11) were male and 12.5% (n=2) were female; followed by 12.5% (n=2) Scotochromogens including one male and other female; and 6.25% (n=1) Rapid grower in a male patient. The rapid grower was identified as *M. vaccae* on basis of growth rate, colony characteristics and biochemical tests. No any photochromogens was isolated during study.

Table 10: Distribution of pulmonary mycobacteriosis on basis of NTM classification

Non-tuberculous mycobacteria	Male		Female		Total	
	No.	%	No.	%	No.	%
Scotochromogens	1	6.25	1	6.25	2	12.50
Non-photochromogens	11	68.75	2	12.5	13	81.25
Rapid growers	1	6.25	0	0.00	1	6.25
Total	13	81.25	3	18.75	16	100.00

5.5. Distribution of drug resistance among culture positive isolates

Among four anti-tubercular drugs, the culture positive mycobacterial isolates were found to be most susceptible to EMB and least susceptible to INH. Of total, 19.38% (n=25) of isolates were resistant to INH, followed by 18.60% (n=24) resistant to RMP and STR and 16.28% (n=21) resistant to EMB.

Table 11: Distribution of drug resistance among culture positive isolates with four anti-tubercular drugs

Drug	Sensitive						Resistance					
	Male		Female		Total		Male		Female		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
INH	76	58.92	28	21.70	104	80.62	16	12.40	9	6.98	25	19.38
RMP	76	58.92	29	22.48	105	81.40	16	12.40	8	6.20	24	18.60
STR	76	58.92	29	22.48	105	81.40	16	12.40	8	6.20	24	18.60
EMB	76	58.92	32	24.80	108	83.72	16	12.40	5	3.88	21	16.28

5.6. Distribution of drug resistance in *M. tuberculosis* isolates

Among 113 culture isolates of *M. tuberculosis* responsible for PTB, only 7.96% (n=9) of *M. tuberculosis* isolates were found to be resistant to at least two of tested four anti-tubercular drugs by proportion method; and all others were sensitive to all four anti-tubercular drugs.

Table 12: Distribution of drug resistance in *M. tuberculosis* isolates

Drug resistance pattern	Male		Female		Total	
	No.	%	No.	%	No.	%
Resistance to H and R only	0	0.00	1	11.11	1	11.11
Resistance to H,R and S	0	0.00	2	22.22	2	22.22
Resistance to H, R, S and E	3	33.33	3	33.33	6	66.67
Total	3	33.33	6	66.67	9	100.00

All 9 resistant *M. tuberculosis* strains were MDR strains resistant to at least INH and RMP. Among them 11.11% (n=1) i.e. one female patient was resistant to INH and RMP only; another 22.22% (n=2) both female were resistant to INH, RMP and STR; and next 66.67% (n=6) with 3 male and 3 female patients were resistant to all four tested anti-TB drugs.

5.7. Distribution of drug resistance in NTM isolates

Among 16 NTM isolates which were isolated from sputum 93.75% (n=15) were resistant to all four drugs whereas one Scotochromogen isolated from a female patient was resistant to INH and STR but sensitive to RMP and EMB.

Table 13: Distribution of drug resistance in NTM isolates

Drug resistance pattern	Male		Female		Total	
	No.	%	No.	%	No.	%
Resistance to H and S only	0	0.00	1	6.25	1	6.25
Resistance to H, R, S and E	13	81.25	2	12.5	15	93.75
Total	13	81.25	3	18.75	16	100.00

5.8. Distribution of mycobacterial infection among patients with family history of TB

Since, chi-square test is highly significant ($P<0.05$), mycobacterial infection and family history of TB are associated.

The association of attributes (Q) =0.46, so there is positive association of Family history of TB with mycobacterial infection.

Table 14: Distribution of mycobacterial infection among patients with family history of TB

Mycobacterial infection	Family history of TB		Total
	Present	Absent	
Yes	70	68	138
No	17	45	62
Total	87	113	200

5.9. Distribution of Mycobacterial infection among smoking patients

Since, chi-square test is insignificant ($P>0.05$), mycobacterial infection and smoking habit are not associated.

Table 15: Distribution of mycobacterial infection among smoking patients

Mycobacterial infection	Smoking		Total
	Yes	No	
Yes	85	53	138
No	33	29	62
Total	118	82	200

5.10. Distribution of Mycobacterial infection among alcoholic patients

Since, Chi-square test is insignificant ($P>0.05$), mycobacterial infection and alcoholism are not associated.

Table 16: Distribution of mycobacterial infection among alcoholic patients

Mycobacterial infection	Alcoholism		Total
	Yes	No	
Yes	69	69	138
No	27	35	62
Total	96	104	200

5.11. Distribution of Mycobacterial infection among patients involved in Animal husbandry

Since, Chi-square test is significant ($P<0.05$), mycobacterial infection and animal husbandry are associated.

The calculated association of attributes (Q) = -0.3. There is negative association between Animal husbandry and mycobacterial infection. The relation may be purely by chance effected by extraneous variable like low population density in village where widely animal husbandry is practiced, and there is less chance of transmission due to less number of infectious cases and less contacts to susceptible cases .

Table 17: Distribution of mycobacterial infection among patients involved in animal husbandry

Mycobacterial infection	Animal husbandry		Total
	Yes	No	
Yes	52	86	138
No	33	29	62
Total	85	115	200

5.12. Distribution of Mycobacterial infection among BCG vaccinated patients

Since, Chi-square test is insignificant (P>0.05), mycobacterial infection and BCG vaccination are not associated.

Table 18: Distribution of mycobacterial infection among BCG vaccinated patients

Mycobacterial infection	BCG vaccination		Total
	Yes	No	
Yes	114	24	138
No	50	12	62
Total	164	36	200

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Mycobacterium is the single genus of Mycobacteriaceae family, consisting of non-motile and non-sporulated acid-alcohol fast rods, and grouped in the supragenic rank of actinomycetes with unusually high 61-71 mole% of G+C in their genomic DNA and presence of high mycolic acids and other lipid contents in their cell wall. The waxy coat confers the idiosyncratic characteristics of the genus, that are acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, distinctive immunological properties and slow growth by restricting the uptake of nutrients (Palomino, 2007). The huge species repertoire exhibit great diversity; many of them freely replicating in natural ecosystem seldom, if ever, involved in disease, to host dependent mycobacteria which cannot replicate in environment that are *M. leprae*, *M. lepraemurium*, *M. avium paratuberculosis* and *M. tuberculosis* complex. Within *M. tuberculosis* complex, *M. tuberculosis*, *M. africanum* and *M. canettii* are primarily pathogenic to human whereas *M. bovis*, *M. microti*, *M. caprae* and *M. pinnipedii* are responsible for zoonotic TB and may occasionally transmit to human (Palomino, 2007). Some NTM species may colonize immunocompetent or immunocompromised individuals in skin, respiratory tract and gastrointestinal tract and some may cause disease called mycobacteriosis (Forbes et al., 2007; Anon, 1989). NTM recovered from humans can be grouped into pulmonary, lymphadenitis, cutaneous and disseminated on the basis of clinical disease they can cause (Wallace, 1990).

Sputum is the material coughed up from the lower respiratory tract and expectorated from the mouth, it contains mucous, cellular debris, microorganisms and possibly blood or pus; the amount, colour and constituents of the mucous are important in the diagnosis of many illnesses, including PTB, pneumonia and lung cancer (Carney, 2002). Bartlett (1974) first suggested clinical laboratories should examine sputum specimens microscopically and refuse to culture specimens with excessive oro-pharyngeal contamination on basis of relative number of squamous epithelial cells, inflammatory cells, and mucus at 100 × magnification. The criteria

developed by Bartlett rejected 17% of specimens for culture but missed fewer potential pathogens so economical for expensive culture of sputum (Wong et al., 1982).

In the present study, the number of patient positive for *Mycobacterium* by either culture or ZN microscopy or fluorescent microscopy were 138 among which 71.01% were male and 28.99% were female which is similar to 69% male and 31% female among 15,468 new smear positive cases recorded during July2008-July2009 affecting mostly 15-45 year age group, i.e. productive age group, so greatly affecting the economic and social status of the country (NTC, 2011). Male population is slightly less than female population as seen in Census 2011 in Nepal but the gender differentiation in TB may be due to exposure of male to external environment or males visiting health centre independently for disease diagnosis but low detection of female TB cases remains a troubling public health issue demanding urgent focused study (Sherchand et al., 2008).

The comparison of ZN staining to the culture, which is regarded as gold standard, showed the sensitivity 90.84%, specificity 91.18%, predictive value of positive test 95.20%, predictive value of negative test 83.78%, percentage of false negatives 9.16% and percentage of false positives 8.82% which showed that ZN staining is quite efficient in the diagnosis of mycobacterial infection in the lung, which is economically viable in low resource setting countries and may be upto village level, where there is more prevalence of TB and other mycobacterial infections and where expensive culture and drug susceptibility may be limited to one of a few reference laboratories in major cities than developed countries where there has been huge breakthrough in the improvement of public health related matters.

The comparison of fluorescence staining to the culture, which is regarded as gold standard, showed the sensitivity 94.57%, specificity 91.54%, predictive value of positive test 95.31%, predictive value of negative test 90.28%, percentage of false negatives 5.43% and percentage of false positive 8.45% which is quite efficient in preliminary disease diagnosis and provides result in few hours as compared to culture taking weeks or up to months to provide result, although culture is useful for the performance of bio-chemical tests to identify the species for definitive disease diagnosis and to provide colonies for the drug susceptibility tests. The lower magnification of 200-250× allows examiner to observe lesser fields than by ZN microscopy for

the diagnosis which made fluorescent microscopy faster and quite useful in those laboratories where many slides are to be observed in a day and results are also required. Although the provision of fluorescent microscope and UV-light source in fluorescent microscopy is more expensive than ZN microscopy, sensitivity and specificity of fluorescent microscopy is greater, so considered superior than ZN microscopy (Laifangbam et al., 2009; Kivihya-Ndugga et al., 2003). Respiratory specimen yields higher smear positivity rates and if more than one specimen is submitted to the laboratory, up to 96% of patients with PTB may be detected by acid fast stains and smear positivity is correlated with the number of colonies recovered in culture (Lipsky et al., 1984). Cross-contamination of the slides during staining and use of contaminated water with saprophytic mycobacteria may yield false positive results during staining (Dizon et al., 1976; Murray et al. 1980)

Among 129 Culture positive cases, 87.60% (n=113) person had TB and 12.40% (n=16) had pulmonary mycobacteriosis. In some laboratories NTM are more commonly isolated from respiratory secretions than *M. tuberculosis* and pulmonary mycobacteriosis are probably underdiagnosed. Pulmonary mycobacteriosis caused by MAC predominate and account for 48-70% of all NTM infections. Significant geographic variability exists both in the prevalence and species responsible for NTM disease (Hornick & Schlesinger, 1998).

Overall 1/3rd of the world's population is currently infected with TB and 5-10% of infected people become actively sick (WHO, 2011). Among 113 culture isolates of *M. tuberculosis* complex responsible for PTB, only 7.96% (n=9) of *M. tuberculosis* isolates were found to be resistant and all resistant *M. tuberculosis* strains were MDR strains resistant to at least INH and RMP. The MDR strains prevalence of TB patients is continually increasing and the MDR prevalence of TB patients never previously treated for TB was 2.9% and among retreatment cases was 11.7% in 2006 (NTC, 2011).

Among 16 NTM isolates, 13 were nonphotochromogens, 2 were scotochromogens and 1 rapid grower identified as *M. vaccae*. 15 of total NTM strains isolated were resistant to all four drugs whereas one Scotochromogen isolated from a female patient was resistant to INH and STR but sensitive to RMP and EMB. Strains of MAC are intrinsically resistant to anti-tuberculosis drugs

and many other antimicrobial agents owing to failure of these drugs to penetrate the lipid rich cell wall (Rastogi et al., 1981; Rastogi et al., 1990). The phenotypic heterogeneity of MAC complicates susceptibility testing; transparent colony types are more resistant than opaque colony types and colony type transition is frequent. Almost all strains of rapidly growing mycobacteria are resistant to anti-tubercular drugs and antimicrobial agent used for treatment depend on identification of the isolate and results of drug susceptibility studies (Nolte & Metchock, 1995).

There may be various risk factors for TB because the number of causal chains is essentially infinite because causation can be expressed on basis of physiological, genetic and behavioural factors; so on the basis of life-cycle of *M. tuberculosis* risk factors may be categorized during infection, progression to disease and adverse outcome of disease (Dye, 2003). Risk factors for infection include spatial proximity to infectious TB patient in household setting or due to work in hospital. Progression to disease may be facilitated by co-morbidities, such as HIV/AIDS, Diabetes or silicosis, as well as by malnutrition (WHO, 2005). Smoking and alcoholism may both be associated with an increased risk for TB through an increased iron content in broncho-alveolar macrophages leading to reduced host defence towards intracellular micro-organisms (Gajalaxmi et al., 2003; Gangaidzo et al., 2001). Adverse outcome is directly or indirectly associated with alcoholism, intravenous drug use, homelessness and malnutrition. Poverty is itself related to a number of above risk factors (WHO, 2005).

In present study, the presence of mycobacterial infection in patients was studied with risk factors family history of TB, smoking, alcoholism, animal husbandry and BCG vaccination. Among these the association between mycobacterial infection and family history of TB and animal husbandry were statistically significant but mycobacterial infection was only positively correlated with family history of TB; it depicts the infectious nature of family member with TB and presence of contacts with TB cases and duration of contacts are important for transmission of disease. The negative correlation of mycobacterial infection and animal husbandry may be due to influence of extraneous variables like more practice of animal husbandry in sub-urban and rural areas where there is comparatively less population density, less air pollution which may be important for transmission and development of lung diseases. Similar to the study of Rijal (2004)

the association of mycobacterial infection with smoking, alcoholism and BCG vaccination was statistically insignificant. The protection status of BCG vaccination seems controversial in the adults.

6.2 Conclusion

Hence, mycobacterial isolates present in the sputum of suspected pulmonary TB patients were distributed and their antibiotic susceptibility pattern assessed.

The association of mycobacterial infection with family history of TB and Animal husbandry was statistically significant, but former has positive association and later has negative association. The association of Smoking, Alcoholism and BCG vaccination with mycobacterial infection was not statistically significant.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

In the present study carried out at NTC from September 2010 to August 2011, sputum samples from 200 suspected new TB patients were selected, according to Barlett's pulmonary specimen culture inclusion criteria, by screening 1500 patients sputum samples. The consent was taken from each of the patients and interviewed for the questionnaire.

Ziehl-Neelsen and Fluorochrome staining was performed with each specimen and then cultured on LJ media after processing by NALC-NaOH method. Among 200 samples selected by Barlett's inclusion criteria, 138 were positive either singly or multiply with ZN staining, Fluorochrome staining or Culture. 125, 128 and 129 positive results were obtained from ZN staining, Fluorochrome staining and Culture respectively. With reference to Culture as Gold standard; sensitivity, specificity, positive predictive value, negative predictive value, false negative and false positive value of ZN staining were estimated as 90.84%, 91.18%, 95.20%, 83.78%, 9.16% and 8.82% respectively. With reference to Culture as Gold standard; sensitivity, specificity, positive predictive value, negative predictive value, false negative and false positive value of Fluorochrome staining were estimated as 94.57%, 91.54%, 95.31%, 90.28%, 5.43% and 8.45% respectively. Fluorochrome staining was found better than ZN staining.

Culture positive isolates were classified into *M. tuberculosis* complex and NTM on the basis of Niacin test, Nitrate reduction test, heat labile Catalase test and growth on 5% PNB incorporated LJ media and possible species were identified. NTM were further classified into Photochromogens, Scotochromogens, Nonphotochromogens and Rapid growers on the basis of growth rate and colony pigmentation. Among 129 culture positive isolates; 87.60% (n=113) belonged to *M. tuberculosis* complex of which 112 were *M. tuberculosis* and 1 was *M. bovis* and 12.40% (n=16) belonged to NTM of which 2 were Scotochromogens, 13 were Nonphotochromogens and 1 Rapid grower identified as *M. vaccae*.

Drug susceptibility testing of culture positive isolates was performed by proportion method. Among 112 M. tuberculosis strains, 9 were resistant to at least INH and RMP, that means all MDR strains, and all other were sensitive to all four antibiotics. Among them, 1 was resistant to INH and RMP only, 2 were resistant to INH, RMP and STR and remaining 6 were resistant to all INH, RMP, STR and EMB. Single M. *bovis* isolate was sensitive to all four antibiotics. Among 16 NTM isolates, 1 Scotochromogen was resistant to INH and STR only, but all other NTM were resistant to all four antibiotics.

The associations of mycobacterial infection with Smoking, Alcoholism and BCG vaccination were found statistically insignificant but that with Family history of TB and Animal husbandry were found statistically significant with positive correlation with Family history of TB and negative correlation with Animal husbandry.

7.2 Recommendations

The following recommendations are made on the basis of present study:

a) *Mycobacterium* species should be identified to the species level for the treatment of mycobacterial diseases.
b) Drug susceptibility testing of each new *Mycobacterium* isolates is recommended.
c) The family contact of infectious TB cases should be considered as high risk individuals.

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APPENDICES

Appendix I: Materials required

A. Bacteriological media

LJ media

B. Chemicals and Reagents

Auramine-Phenol solution

Carbol-Fuchsin solution

20% Sulfuric acid

0.1% Methylene blue

NALC-NaOH Solution

0.067 M Phosphate buffer

Mc Farland 1 standard

Isoniazid

Rifampicin

Absolute Methanol

95% Ethanol

Dihydrostreptomycin sulfate

Ethambutol

4% Aniline in Ethanol

10% Cyanogen bromide

30% Hydrogen peroxide

10% Tween-80

Para-Nitrobenzoic acid

Acetone

Dimethyl formamide

Nitrate substrate broth

50% Hydrochloric acid

0.2% Sulfanilamide

0.1% N-Naphthylethylene-diamine

Zinc dust

Distilled water

C. Equipments and Instruments

Biological safety cabinets, Class IIA	AIR TECH, Japan; Dalton, Japan
Fluorescent microscope	OPTIKA, Japan
Light microscopes	OLYMPUS, Japan
Autoclaves	Ovalace, ALP, Life Japan
Incubators	R.K.I. IKEMOTO, Japan; ALP, Japan
Hot air ovens	KEF 45D, Japan
Coagulators/Inspissators	HIRASAWA TE-HCR, Japan
Refrigerated centrifuge	TOMY LX-130, Japan
Refrigerators	National, Japan
Distillation apparatus	Kayagaki, Japan
Bunsen burners	
Vortex mixer	
Digital balances	
Water taps	
Water bath	Yorco, India
Heater	
Magnetic stirrer	
Pipette fillers	
Slide driers	
Thermometers	

D. Glassware

Screw capped culture tubes

Test tubes

Conical flasks

Beakers

Measuring cylinders

Pipettes

Pasture pipettes

Funnels

Microscopic slides

E. Miscellaneous

Inoculating loops

Rubber teats

Sputum containers

Centrifuge tubes

Gloves

Tube holders

Centrifuge tube holders

Slides holders

Metal vessels

Staining tray

Slide racks

Glass beads

Cotton

Iron rods

Droppers

Polythene bags

Slanting trays

Filter papers

Masks

Aluminium foils

Tripod stands

Pipette filler

Immersion oils

Lens papers, etc.

Appendix II: Bacteriological media

A. Lowenstein Jensen (LJ) medium (WHO, 1998)

LJ medium is the most widely used for mycobacterial culture and LJ medium containing glycerol favours the growth of *M. tuberculosis* while that without glycerol but containing sodium pyruvate, in instead, encourages the growth of *M. bovis*.

- **Ingredients**

Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade)	12ml
Distilled water	600ml

The ingredients were dissolved in order in the distilled water by heating; and autoclaved at 121ºC for 30 minutes to sterilize, and then cooled to room temperature.

Malachite green solution, 2%

Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques, the dye was dissolved in sterile distilled water by placing the solution in the incubator for 1-2 hours to make fresh solution, to avoid precipitated or changed to a less-deeply coloured solution.

Homogenized whole eggs

Fresh hens' eggs, not more than seven days old, were cleaned by scrubbing thoroughly with a hand brush in detergent containing warm water and then rinsed thoroughly in running distilled water. Then the eggs were soaked in 70% ethanol for 15 minutes and dried in blotting paper. Before handling these clean dry eggs, the hands of

the handler had been scrubbed and thoroughly washed with soap water. The eggs were cracked into a sterile flask containing sterile glass beads and then stirred on magnetic stirrer to make homogenized whole eggs. The eggs homogenate was filtered though sterile metal screen to separate non homogenized egg portions.

- **Preparation of complete medium**

The following ingredients were aseptically poured into large sterile flask and mixed well.

Mineral salt solution	600ml
Malachite green solution	20ml
Homogenized eggs (20-25 eggs, depending on size)	1000ml

The complete egg medium was distributed in 5ml volumes with sterile pipettes into 130×15mm sterile screw- capped test tubes. The tubes were slanted on the slanting racks to make appropriate slope on the media and then inspissated within 15 minutes to prevent sedimentation of the heavier ingredients.

- **Coagulation of medium**

Before loading, the inspissator was pre-heated to 80 ºC, then the bottles were placed in a slanted position in the inspissator and the medium was coagulated for 45 minutes at 80-85 ºC. Heating for longer time period or at higher temperature may affect the quality of the medium. Discolouration of medium, or appearance of the holes or bubbles on the surface of the medium indicates faulty coagulation procedures, and poor quality media were discarded.

- **Sterility check**

After inspissation, the whole media batch or a representative sample thereof was incubated at 35-37 ºC for 24 hours and checked for the growth of contaminants as sterility control.

- **Storage**

  The LJ medium was dated and stored in the refrigerator at 4 ºC. For optimum isolation from specimens, LJ medium should not be older than 4 weeks, but tightly closed medium prevented from drying can work after several weeks.

B. LJ medium containing primary anti-tubercular drugs

The primary anti-tubercular drugs are incorporated, in their solutions, into the complete LJ broth and homogenously mixed before inspissation step. The critical concentrations of the drugs are maintained in the drug containing media to assess the drug susceptibilities of the test mycobacterial cultures by proportion method. Several graded concentrations of anti-tubercular drugs are made for quality control of anti-tubercular drugs using standard laboratory *M. tuberculosis* H37Rv strain.

a. Isoniazid (INH/H)

For dry and pure INH, the correction factor is 1.

Solution I: 10.0 mg INH dissolved in 50 ml sterile water (200µg/ml)

Solution II: 2.5 ml Solution I, made up to 25ml with sterile water (20 µg/ml)

Solution III: 5.0 ml Solution II, made up to 10ml with sterile water (10 µg/ml)

Stock solution I (200µg/ml) can be aliquoted into sterile cryovials and stored frozen at -20 ºC.

Final drug concentration in media (µg/ml)

	0.2 µg/ml	0.1 µg/ml	0.05 µg/ml	0.025 µg/ml
Medium (ml)	247.5	19.8	19.8	19.8
Solution II (ml)	2.5	_	_	_
Solution III (ml)	_	0.2	0.10	0.05
Water (ml)	_	_	0.10	0.15
Final volume(ml)	250	20	20	20

b. Rifampicin (RMP/R)

The correction factor is usually 1 for pure RMP or 1.03 for the sodium salt

Solution I:

Factor 1: 40.0 mg RMP, dissolved in 10.0 ml dimethyl sulfoxide (4000 µg/ml)

Factor 1.03: 41.2 mg RMP, dissolved in 10.0 ml sterile water (4000 µg/ml)

Solution II: 2.5 ml Solution I, made up to 10.0 ml with sterile water (1000 µg/ml)

Final concentration in drug media (µg/ml)

	40 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml
Media (ml)	247.5	19.8	19.8	19.8
Solution I (ml)	2.5	_	_	_
Solution II (ml)	_	0.2	0.10	0.05
Water (ml)	_	_	0.10	0.15
Final volume (ml)	250	20	20	20

c. Streptomycin (STR/S)

For Dihydrostreptomycin sulfate dry and pure, the correction factor is 1.251. For Dihydrostreptomycin sulfate, dry and 98% pure (which is common), the factor is 1.277.

Solution I:

Factor 1.251: 12.51 mg Dihydrostreptomycin sulfate dissolved in 25 ml sterile water (400µg/ml)

Factor 1.277: 12.77 mg Dihydrostreptomycin sulfate dissolved in 25 ml sterile water (400µg/ml)

Solution II: 5 ml Solution I, made up to 10 ml with sterile water (200 µg/ml)

Stock solution I (400µg/ml) can be aliquoted into sterile cryovials and stored frozen at -20 ºC.

Final concentration in drug media (µg/ml):

	4 µg/ml	2 µg/ml	1 µg/ml	0.5 µg/ml
Media (ml)	247.5	19.8	19.8	19.8
Solution I (ml)	2.5	_	_	_
Solution II (ml)	_	0.2	0.10	0.05
Water (ml)	_	_	0.10	0.15
Final volume (ml)	250	20	20	20

d. Ethambutol (EMB/E)

For Ethambutol dihydrochloride, the correction factor is 1.36.

Solution I: 13.6 mg Ethambutol dihydrochloride dissolved in 50 ml sterile water (200µg/ml)

Solution II: 2.5 ml Solution I, made up to 10 ml with sterile water (50 µg/ml)

Stock solution I (200µg/ml) can be aliquoted into sterile cryovials and stored frozen at -20 ºC.

Final concentration in drug media (µg/ml):

	2 µg/ml	0.5 µg/ml	0.25 µg/ml	0.125 µg/ml
Media (ml)	247.5	19.8	19.8	19.8
Solution I (ml)	2.5	_	_	_
Solution II (ml)	_	0.2	0.10	0.05
Water (ml)	_	_	0.10	0.15
Final volume (ml)	250	20	20	20

C. LJ medium containing para-Nitrobenzoic acid (PNB)

The PNB is incorporated, in its solution, into the complete LJ broth and homogenously mixed before the inspissation step to make its final concentration in the media as 500µg/ml.

75mg of PNB dissolved in 1-2 ml of Acetone.

The solution was made up to 150 ml with LJ medium.

Appendix III: Chemicals and Reagents

a. Auramine-Phenol solution (0.1%)

Auramine powder 1g/l

Phenol (melted) 30ml/l

95% Ethanol 100ml/l

Distilled water up to 1000 ml

1g of Auramine powder was dissolved in 100 ml of 95% Ethanol. Phenol was melted by heating in water bath, and 30ml was added to Auramine solution. Then the final volume was made up to 1000 ml by adding distilled water, mixed well and stored in refrigerator.

b. Carbol-Fuchsin solution (1%)

Basic Fuchsin 10g/l

Phenol (melted) 60ml/l

95% Ethanol 100ml/l

Distilled water up to 1000 ml

10 g of basic fuchsin powder was dissolved in 100 ml of 95% Ethanol. Phenol was melted by heating in water bath, and 60 ml was added to fuchsin solution. Then the final volume was made up to 1000 ml by adding distilled water, mixed well and filtered and stored in amber coloured bottle.

c.20% Sulfuric acid solution

Concentrated Sulfuric acid 200ml/l

Distilled water 800ml

200 ml of concentrated Sulfuric acid was carefully added, with constant mixing, to 800ml of cold water in a flask kept in a sink with water for cooling, and then stored.

d.0.1% Methylene blue

Methylene blue power 1g/l

Distilled water 1000ml

1g of Methylene blue powder was dissolved in distilled water and final volume was made to 1 litre by adding distilled water, and then stored.

e. NALC-NaOH preparation:

The digestant, decontaminant mixture was prepared using distilled water using three solutions:

- 1N (4%) NaOH (50 ml)
- 0.1M (2.94%) trisodium citrate.$3H_2O$ (50ml)
- NALC powder (0.5g)

NaOH and citrate solutions were mixed, sterilized and stored in sterile screw capped flasks for later use.

After addition of NALC, the solution was used within 24 hours.

f. 0.067M phosphate buffer

Solution A: 9.47g of Na_2HPO_4 was dissolved in 1 litre of distilled water.

Solution B: 9.07g of KH_2PO_4 was dissolved in 1 liter of distilled water.

50 ml of solution A was mixed with 50 ml of solution B to obtain 0.067M phosphate buffer of pH 6.8.

g. Mc Farland 1 standard

1g of anhydrous Barium chloride was dissolved in 100ml of distilled water to make 1% $BaCl_2$ solution.

1ml of concentrated sulfuric acid was mixed with 100ml of distilled water to make 1% H_2SO_4 solution.

0.1 ml of 1% $BaCl_2$ solution was added to 9.9 ml of 1% H_2SO_4 solution to make Mc Farland 1 turbidity standard.

h. 4% Aniline solution

Fresh, clear colourless Aniline	4ml
95% Ethanol	96ml

Wearing gloves, 4ml of Aniline was mixed to 96ml of 95% Ethanol in an amber bottle and stored in dark in refrigerator. Aniline is oncogenic and penetrates through the skin, so Aniline was handled carefully and yellow discoloured solutions were discarded.

i. 10% Cyanogen bromide solution

Cyanogen bromide crystals 5g

Distilled water 50ml

Cyanogen bromide is a severe lacrimator, toxic if inhaled, oncogenic and penetrates through skin. Wearing the gloves and all operation performed inside BSC, 5g of Cyanogen bromide crystals were carefully added to 50ml of distilled water in a glass beaker, and left to dissolve, covering the beaker with Aluminium foil, for about 24 hours at room temperature; and then poured into tightly capped amber bottle and stored in refrigerator. The solution should not be heated because toxic gas releases, and Bunsen burner should be extinguished while working with this solution in BSC. In acidic solution, Cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic so all reaction tubes were made alkaline by adding Sodium hydroxide before discarding.

j. Sodium nitrate substrate in phosphate buffer

0.01M Sodium nitrate was prepared in 0.022M phosphate buffer, pH 7.0 as follows:

KH_2PO_4 3.02g

Distilled water 1000ml

Potassium dihydrogen phosphate was dissolved in distilled water to make 0.022M solution........................Solution 1

Na_2HPO_4 3.16g

Distilled water 1000ml

Disodium hydrogen phosphate was dissolved in distilled water to make 0.022M solution

.....................................Solution 2

611ml of Solution 2 was added to 389 ml of Solution 1, and mixed well, to make phosphate buffer 0.022M and pH maintained at 7...................Solution 3

$NaNO_3$ 0.85g

Solution 3 1000ml

The sodium nitrate was dissolved in phosphate buffer to make volume 1000ml, to make complete Sodium nitrate substrate buffer. It was dispensed in 100ml aliquots and sterilized by autoclaving at 121 ºC for 15 minutes.

k. 50% Hydrochloric acid solution

Conc. HCl 10ml

Distilled water 10ml

Conc. HCl was slowly added to distilled water and stored in an amber bottle in the dark in the refrigerator.

l. 0.2% Sulfanilamide solution

Sulfanilamide 0.2%

Distilled water 100ml

Sulfanilamide was dissolved in distilled water and stored in an amber bottle in the dark in a refrigerator.

m. 0.1% N-naphthylethylene-diamine solution

N-naphthylethylene-diamine 0.1g

Distilled water 100ml

Naphthylethylene-diamine was dissolved in distilled water and stored in an amber bottle in the dark in a refrigerator.

n. 30% Hydrogen peroxide solution

Hydrogen peroxide 30ml

Distilled water 70ml

Hydrogen peroxide was mixed with distilled water to make 100ml, autoclaved at 121 ºC for 15 minutes and stored in refrigerator.

o. 10% Tween 80

Tween 80 10ml

Distilled water 90ml

Tween 80 was mixed with distilled water and autoclaved at 121 ºC for 15 minutes. After autoclaving if Tween settled it was resuspended immediately during cooling and stored in the refrigerator.

Appendix IV: Questionaire

Patient code No:

Date:

Name:

Address:

Age:

Sex:

1. Suspected Case: a. New ☐ b. Follow up ☐ c. Relapse ☐ d. Default ☐ e. MDR ☐

2. Family history of Tuberculosis: a. Present ☐ b. Absent ☐

3. Habit of smoking: a. Yes ☐ b. No ☐

4. Habit of alcoholism: a. Yes ☐ b. No ☐

5. Presence of other immune-compromising or debilitating diseases: a. Yes ☐ b. No ☐
   If yes then, a. AIDS ☐ b. Cancer ☐ c. Diabetes ☐ d. Others ☐

6. Practice of animal husbandry: a. Yes ☐ b. No ☐

7. BCG immunization: a. Yes ☐ b. No ☐

8. Presence of symptoms of Tuberculosis: a. Yes ☐ b. No ☐
   If yes then, a. Fever ☐ b. Chest pain ☐ c. Cough ☐ d. Hemoptysis ☐
   e. Weight loss ☐ f. Others ☐

Appendix V: Consent letter (In Nepali)

मञ्जुरीपत्र

म त्रिभूवन विश्वविधालय र राष्ट्रिय क्षयरोग केन्द्रको समन्वयमा, राण्ट्रिय क्षयरोग केन्द्रमा हुन गइरहेको क्षयरोग संग सम्बन्धित किटाणुको बारेमा अनुसन्धानात्मक कार्यको लागि मेरो खकारका केही नमुनाहरु उपलब्ध गराउन र रोगसंग सम्बन्धित सोधिएका केही प्रश्नहरुको उत्तर दिन पनि म मञ्जुरी जनाउंछु ।
यो अनुसन्धानमा सहभागी हुंदा मेरो रोग पहिचान तथा उपचारमा खासै फरक नपारेपनि यो अस्पतालको उपचारात्मक नीतिमा सकारात्मक योगदान पुऱ्याउनेछ भन्ने मैले विश्वास लिएको छु।
मेरो नाम एउटा कोड नम्बरको रुपमा मात्र प्रयोग हुने भएकोले अनुसन्धानकर्ता बाहेक अरु कसैलाई पनि मेरो रोग र अवस्थाबारे थाहा हुने छैन र मैले यो अनुसन्धानमा सहभागिता केहि उपहारको अपेक्षा राखेर नभइ विशुद्ध अध्ययन तथा अनुसन्धानका लागि जनाएको हुं।
मैले नचाहेको खण्डमा यो अनुसन्धानमा भाग नलिन अथवा बिचैमा सहभागिता हटाउन पनि सक्दछु।

सहभागिको कोड नम्बर ....
सहभागिको नाम..
सहभागिको ठेगाना..
सहभागिको दस्तखत...................................
मिति...........................

Appendix VI: Recording and reporting of laboratory results

a. Bartlett criteria for judging acceptability of sputum specimen (Bartlett, 1974)

Method	Values	Criteria for acceptability
Stained smears are examined under 100× magnification and the number of Squamous epithelial cells (EPI) or White blood cells (WBC) per field is determined	+ and – values are assigned: +2 if >25 WBC +1 if 10-25 WBC +1 if mucus seen -2 if >25 EPI -1 if 10-25 EPI	Any positive score (Sum of positive and negative assigned)

b. Reporting of ZN staining (RNTCP, 1997)

Number of AFB	Number of fields to be examined	Result	Grading
No AFB in 100 OIF*	100	Negative	
1 to 9 AFB per 100 OIF	200	Scanty	Exact number seen recorded
10 to 99 AFB per 100 OIF	100	Positive	1+
1 to 10 AFB per OIF	50	Positive	2+
More than 10 AFB per OIF	20	Positive	3+

*Oil Immersion Field

c. Reporting of Fluorochrome staining (200-250Î magnification, 1 length = 30 fields = 300 High Power Fields) (NTC, 2010)

Number of AFB	Number of fields to be examined	Result	Grading
No AFB in 200Î field	30 fields	Negative	
1 to 29 AFB in 200Î field	30 fields	Scanty	Exact number seen recorded
30 to 299 AFB in 200Î field	30 fields	Positive	1+
10 to 100 AFB in 200Î field	1 field	Positive	2+
More than 100 AFB in 200Î field	1 field	Positive	3+

d. Reporting of Culture (WHO, 1998)

Reading	Report
No growth	Negative
1 to 19 colonies	Scanty (Exact number of colonies reported)
20 to 100 colonies	Positive (1+)
100 to 200 colonies	Positive (2+)
200 to 500 colonies (almost confluent growth)	Positive (3+)
More than 500 colonies (confluent growth)	Positive (4+)
Contaminated	Contaminated

Appendix VII: Sample data table (Questionaire and experimental)

Code No.	Sex	Age	Staining		Culture	Biochemical tests				Organism	Drug susceptibility tests				Risk factors				
			Ziehl-Neelsen	Fluorochrome		Growth on PNB	Niacin	68°C labile Catalase	Nitrate Reduction		Isoniazid	Rifampicin	Streptomycin	Ethambutol	TB family history	Smoking	Alcoholism	Animal husbandry	BCG vaccination
N1	M[l]	38	-	1+	2+	-	+	-	+	Mt[b]	S[f]	S	S	S	P[h]	Y[j]	Y	Y	Y
N2	F[m]	50	-	-	C[a]										A[i]	N[k]	N	N	Y
N3	M	33	-	-	-										P	N	Y	N	Y
N4	F	36	-	-	C										A	N	N	Y	Y
N5	M	67	-	-	-										A	Y	N	Y	N
N6	M	26	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N7	M	76	-	-	-										A	N	N	Y	N
N8	M	49	-	-	-										A	Y	Y	Y	N
N9	F	30	2+	3+	13 Col	-	+	-	+	Mt	R[g]	R	R	R	A	N	N	N	Y
N10	M	69	-	-	-										A	Y	Y	Y	Y
N11	M	74	1+	1+	3+	+	-	+	+	Mv[c]	R	R	R	R	A	Y	N	Y	N
N12	M	62	-	-	-										A	Y	Y	Y	Y
N13	F	54	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	N
N14	F	23	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N15	F	43	-	1+	1+	+	-	+	+	SC NTM[d]	R	S	R	S	P	N	N	Y	Y
N16	F	25	2/100	1+	2+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	

N17	M	55	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N18	F	18	-	-	-										A	N	N	N	Y
N19	M	14	-	-	-										A	N	N	Y	Y
N20	M	50	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N21	M	50	-	1+	-										A	Y	Y	N	Y
N22	M	39	1+	1+	-										P	Y	Y	Y	Y
N23	M	64	1+	1+	1+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N24	M	57	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N25	M	50	-	-	-										A	Y	Y	Y	Y
N26	M	21	-	-	-										A	Y	N	N	Y
N27	M	52	-	-	-										A	Y	Y	Y	Y
N28	F	20	-	-	-										A	N	N	Y	N
N29	M	50	-	-	-										A	Y	Y	Y	Y
N30	M	65	-	-	-										A	Y	Y	N	Y
N31	M	60	-	-	-										P	Y	Y	Y	Y
N32	M	63	-	-	-										A	Y	N	Y	Y
N33	M	28	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N34	F	23	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	N
N35	F	37	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	N	Y	Y
N36	M	53	2+	2+	3 Col	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N37	M	40	2+	2+	1+	-	+	-	+	Mt	S	S	S	S	A	N	Y	Y	Y
N38	F	32	3+	3+	1+	-	+	-	+	Mt	S	S	S	S	A	Y	N	Y	N
N39	M	28	3+	3+	1+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y

N40	F	32	-	-	-										P	N	N	Y	N
N41	M	23	-	-	-										A	Y	Y	N	Y
N42	M	35	-	-	C										P	Y	Y	Y	N
N43	M	42	2+	1+	1+	-	+	-	+	Mt	S	S	S	S	A	N	Y	Y	Y
N44	F	46	-	-	-										P	Y	N	Y	Y
N45	M	65	-	-	-										A	Y	Y	Y	N
N46	M	19	3+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	N
N47	F	44	-	-	-										P	Y	N	N	Y
N48	M	24	-	-	-										P	N	N	Y	Y
N49	F	77	1/100	-	1+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N50	F	35	-	-	C										A	Y	N	N	Y
N51	M	22	1/100	1+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	N
N52	M	38	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N53	M	19	-	-	-										A	Y	Y	N	N
N54	M	36	3+	1+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N55	M	55	-	-	5 Col	+	-	-	-	NC NTM[e]	R	R	R	R	A	Y	N	N	Y
N56	M	58	-	-	-										A	Y	Y	N	Y
N57	M	40	-	-	-										A	Y	Y	N	Y
N58	M	17	1+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N59	M	16	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N60	M	55	-	-	-										A	Y	Y	Y	Y
N61	M	17	-	-	7 Col	+	-	+	-	NC NTM	R	R	R	R	A	N	N	N	Y
N62	M	33	-	-	6 Col	+	-	+	-	NC NTM	R	R	R	R	A	Y	Y	N	Y

N63	F	25	3+	3+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N64	M	30	-	-	-										A	Y	N	N	Y
N65	F	34	-	-	15 Col	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N66	F	24	-	-	-										P	N	N	Y	N
N67	M	49	-	1+	2+	+	-	+	-	NC NTM	R	R	R	R	A	N	N	Y	N
N68	M	40	-	-	-										A	N	N	Y	Y
N69	M	28	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N70	M	60	-	-	-										A	Y	Y	Y	Y
N71	M	20	-	-	-										P	N	N	N	Y
N72	M	66	-	-	-										A	Y	Y	Y	N
N73	M	25	-	-	-										A	N	N	N	Y
N74	M	37	-	-	-										P	Y	N	Y	Y
N75	M	48	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N76	M	27	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	N
N77	M	40	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N78	F	42	1/100	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N79	M	39	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N80	F	35	2/100	1+	2+	-	+	-	+	Mt	S	S	S	S	A	Y	N	Y	N
N81	M	28	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N82	M	50	3+	3+	3+	-	+	-	+	Mt	R	R	R	R	A	Y	Y	Y	Y
N83	M	40	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N84	M	60	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N85	M	30	-	-	-										A	N	Y	Y	Y

N86	M	24	-	1+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N87	F	17	2+	3+	3+	-	+	-	+	Mt	R	R	R	R	P	N	N	Y	Y
N88	M	38	2/100	-	-										A	N	N	Y	Y
N89	M	20	-	-	-										A	N	N	N	Y
N90	F	30	1/100	-	-										P	N	N	N	Y
N91	F	46	-	-	-										A	N	Y	N	Y
N92	F	20	-	-	-										A	N	N	N	Y
N93	M	49	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N94	M	39	2+	1+	3+	+	-	+	+	SC NTM	R	R	R	R	P	Y	Y	Y	Y
N95	F	20	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N96	F	42	-	-	C										P	Y	Y	N	N
N97	M	37	2/100	1+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N98	M	55	-	-	-										A	Y	Y	N	Y
N99	M	17	-	-	-										P	N	N	N	Y
N100	M	24	-	-	-										P	N	N	N	Y
N101	M	62	-	-	-										A	Y	Y	Y	Y
N102	M	54	-	-	2 Col	+	-	-	-	NC NTM	R	R	R	R	A	Y	N	N	Y
N103	M	30	2+	1+	3+	+	-	-	-	NC NTM	R	R	R	R	A	Y	Y	Y	Y
N104	F	23	2+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N105	M	20	1+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	N	Y	Y	Y
N106	F	22	4/100	1+	3+	-	+	-	+	Mt	R	R	R	S	P	N	N	N	Y
N107	M	25	-	-	-										A	Y	N	Y	Y
N108	F	38	-	-	-										A	N	N	Y	Y

N109	M	21	1/100	1+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N110	F	25	-	-	-										A	N	N	N	Y
N111	M	67	1+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N112	F	50	1+	1+	C										A	Y	Y	N	N
N113	M	33	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	N	N	Y
N114	M	46	2+	1+	C										A	Y	Y	N	Y
N115	F	37	3+	3+	3+	+	-	-	-	NC NTM	R	R	R	R	P	N	N	N	Y
N116	M	50	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N117	M	38	1+	1+	3+	+	-	+	-	NC NTM	R	R	R	R	P	Y	Y	N	Y
N118	M	35	1+	1+	2+	+	-	+	-	NC NTM	R	R	R	R	P	N	N	N	Y
N119	F	25	2+	2+	3+	+	-	+	-	NC NTM	R	R	R	R	P	N	N	N	Y
N120	F	53	-	-	-										P	Y	N	Y	N
N121	M	48	3+	2+	3+	+	-	-	-	NC NTM	R	R	R	R	P	Y	Y	Y	Y
N122	M	47	-	-	2+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N123	M	34	-	1+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N124	M	31	3+	3+	3+	+	-	-	-	NC NTM	R	R	R	R	P	N	Y	Y	Y
N125	F	40	5/100	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	Y
N126	M	54	2+	1+	3+	+	-	+	-	NC NTM	R	R	R	R	A	Y	N	Y	Y
N127	M	36	-	-	-										P	N	N	Y	Y
N128	F	18	2+	1+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N129	F	27	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N130	F	19	3+	3+	3+	-	+	-	+	Mt	R	R	R	R	P	N	N	N	Y
N131	M	74	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N

N132	M	47	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N133	M	38	-	-	-										A	N	Y	N	Y
N134	M	50	2+	1+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N135	M	34	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N136	M	35	3+	2+	C										P	Y	Y	Y	Y
N137	M	47	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N138	M	24	-	-	-										A	N	N	N	Y
N139	F	35	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	N	Y
N140	M	66	2+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	N	Y	Y
N141	F	43	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N142	M	57	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N143	M	39	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N144	M	25	-	-	-										A	N	N	N	Y
N145	F	24	8/100	1+	-										P	N	N	N	Y
N146	M	31	4/100	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N147	M	67	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	N
N148	F	22	-	-	-										A	N	N	Y	Y
N149	M	39	2+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N150	M	54	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N151	M	72	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	N
N152	F	42	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N153	M	25	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N154	M	69	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y

N155	M	75	-	1+	1+	-	+	-	+	Mt	S	S	S	S	A	Y	N	N	N
N156	M	38	1+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N157	M	50	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N158	M	35	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N159	M	55	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N160	F	18	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	Y
N161	F	70	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	Y	N
N162	M	46	-	-	-										A	Y	Y	Y	Y
N163	F	45	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N164	F	25	3+	2+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N165	M	72	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N166	M	25	3+	1+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N167	M	35	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	Y	Y	Y
N168	M	27	2+	1+	10 Col	-	+	-	+	Mt	S	S	S	S	P	Y	Y	N	Y
N169	M	58	1+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N170	M	22	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	Y
N171	M	52	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N172	M	42	-	-	-										P	Y	Y	Y	Y
N173	M	22	2/100	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	Y
N174	M	65	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	N	N	N
N175	M	22	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N176	M	22	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N177	F	22	2+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y

N178	F	26	-	-	-										P	N	N	N	Y
N179	F	60	3+	1+	3+	-	+	-	+	Mt	S	S	S	S	A	N	N	Y	N
N180	M	52	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	Y
N181	F	20	2/100	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N182	M	60	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	Y	N
N183	M	21	3+	2+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N184	F	25	3+	3+	3+	-	+	-	+	Mt	R	R	S	S	P	N	N	N	Y
N185	M	32	3+	3+	3+	-	+	-	+	Mt	R	R	R	R	A	Y	Y	N	Y
N186	M	38	3+	3+	3+	-	+	-	+	Mt	R	R	R	R	A	Y	Y	N	Y
N187	F	25	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N188	M	24	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N189	M	18	3+	3+	2+	-	+	-	+	Mt	S	S	S	S	P	N	N	Y	Y
N190	F	24	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	N	N	N	Y
N191	F	21	-	-	-										A	N	N	N	Y
N192	M	32	3+	3+	2+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N193	M	52	-	-	-										A	Y	Y	N	Y
N194	M	23	2/100	-	-										A	Y	Y	N	Y
N195	M	17	-	-	-										A	N	N	N	Y
N196	F	22	1+	1+	2+	-	+	-	+	Mt	R	R	R	S	P	N	N	N	Y
N197	M	24	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N198	M	35	2+	2+	2+	-	+	-	+	Mt	S	S	S	S	A	Y	Y	N	Y
N199	M	49	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y
N200	M	35	3+	3+	3+	-	+	-	+	Mt	S	S	S	S	P	Y	N	N	Y

[a] Contamination
[b] *M. tuberculosis*
[c] *M. vaccae*
[d] Scoto-chromogenic NTM
[e] Nonchromogenic NTM
[f] Sensitive
[g] Resistant
[h] Present
[i] Absent
[j] Yes
[k] No
[l] Male
[m] Female