# CHAPTER-I

## 1 INTRODUCTION

Tuberculosis is a chronic infectious disease regulated almost entirely by the cell-mediated immune response (CMI) of the host against *M. tuberculosis*. It is a world wide disease estimated to affect approximately thirty million people (IUAT/WHO, 1992) and WHO has declared tuberculosis as a global emergency in 1993 (Keats, 1996).

In the last decade, tuberculosis has reemerged as one of the leading cause of death (nearly 3 million deaths annually). The estimated 8.8 million new cases every year corresponds to 52,000 deaths per week or more than 7,000 deaths each day which translate into more than 1,000 new cases every hour, every day. These death rates, however only partially depict the global tuberculosis threat, more than 80% of tuberculosis patients are in the economically productive age of 15 to 49 years. The emergency of AIDS and decline of socioeconomic standard contribute to the disease's resurgence in industrialized countries. In most developing countries, although the disease has always been endemic, its severity has increased because of the global HIV pandemic and extensive social restructuring due to rapid industrialization and conflicts (Foulds and Brien, 1998).

The global incidence of tuberculosis estimated 8.8 million cases world wide, more than 40% are in south-east Asia; i.e. WHO has estimated that in three people in the world are infected with tuberculosis that comprises 1.7 billion people, 95% of them living in developing countries (Rabiglone *et al*, 1995).

Tuberculosis is a major health problem in the SAARC region causing an immense burden of disease. WHO estimate that more than 3 million new tuberculosis cases occur in the region with about 1 million deaths every year due to this serious but curable

disease. This situation will worsen further with TB/HIV co-infection and MDR tuberculosis. The estimated incidence of TB cases for 1995 was 3.1 million in SAARC region which represented about 40% of the global burden of the disease. The incidence rate of all form of tuberculosis in 1995 was estimated to 241-254 per 1, 00,000 of population (Kumar and Bam, 1996).

About 60% of the adult population has been infected with the tuberculosis bacilli in Nepal. Every year 2,00,000 develops tuberculosis and 13,000 -15,000 die, one infected patient can transmit the disease to 10-15 people every year and at least 5% of the newly diagnosed patient with tuberculosis have resistance one or more drugs (Sharma and Smith, 1996).

In Nepal, 45% of the total population is infected with TB, out of which 60% are in the productive age group (15-45). Every year 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. These 20,000 are able to spread the disease to others. Tuberculosis cases are scattered all over the country and the prevalence is higher in urban and densely populated region. DOTS strategy was started in April 1996 in Nepal (only in four districts), but from April 2001, DOTS was implemented throughout the country and reduced the number of deaths from 5,000 to 7,000. By July 2003 DOTS had been expanded to 335 treatment centers with 1407 sub centers of the country with 94% of the population coverage by DOTS. The treatment success rate in DOTS is now around 90% (NTP, Nepal, 2004).

*Mycobacterium* is non-motile, non-sporing, non-capsulated and mostly very slow growing bacilli. They are difficult to stain due to presence of waxy material in their cell wall. They are stained by hot carbol fuschin or auramine dye and once stain; they resist decolonization by dilute acid and therefore refer to as AFB. The genus *Mycobacterium* includes pathogenic and saprophytic species. There are over 60 well defined species. Four very closely related species are responsible for mammalian TB are *M. tuberculosis* (human tubercle bacilli), *M. bovis* (bovine tubercle bacilli), *M. microti*

(vale tubercle bacilli) and *M. africanum* (mainly found in equatorial Africa), and all are classified as *M. tuberculosis* complex.

The most important *Mycobacterium* species, which causes tuberculosis, is *M. tuberculosis*, main reservoir being infected human. The most common site for infection is lung and glands, bones, joints, brain and meninges and other organs may be affected. Most infection with *M. tuberculosis* is caused by inhaling droplets or dust particles containing the bacilli.

Due to the risk of the spread of disease and the potential for the emergence of drug resistant strains, the rapid diagnosis of the TB is very important. Concerning detection of TB, the lab diagnosis of TB relies on direct smear examination of sputum by Ziehl-Neelsen or Auramine fluorochrome stain and culture. It is also aided by the chest X-ray. Microbial examination of smear of AFB by Z-N stain is currently the most rapid method for the detection of *M. tuberculosis* but its sensitivity is low i.e. required at least 10,000 bacterial cells per ml of sputum and also none specific, but auramine staining method has higher sensitivity than that of the Z-N stain but there are chances of false positive. Isolation of the organism by culture and identification by bio-chemical test is much more sensitive and specific but is time consuming (Chakraborty, 2003).

The single most important risk factor for developing tuberculosis is now HIV infection in Nepal. Majority of people with AIDS is the first stages of the epidemics have been commercial sex worker women. The increases in TB cases in women are because of AIDS in female (Smith, 1996).

Tuberculosis has remained as a major health problem in the developing countries like Nepal. For case finding different methods like Ziehl-Neelsen stain, Auramine Fluorochrome, culture etc were used but no any single method was sufficient for identifying person suffering from tuberculosis. Efficacy of each technique is different. However, in Nepal there is no such type of information regarding efficacy of different

technique. In this regard, this study aims to compare the different diagnostic methods for pulmonary tuberculosis.

# CHAPTER-II

## 2 OBJECTIVES

### 2.1 General objective

To study the correlation between chest X-ray, direct sputum smear examination by Ziehl-Neelsen stain, Auramine fluorochrome stain and sputum culture for *M. tuberculosis*.

### 2.2 Specific objectives

1. To examine the direct sputum smear by Ziehl-Neelsen stain, Auramine fluorochrome stain.

2. To find out the correlation between chest X-ray, direct sputum smear examination by Ziehl-Neelsen stain, Auramine fluorochrome stain and sputum culture for MTB.

3. To isolate and identify MTB from sputum.

4. To find out the prevalence of TB in different age and sex.

# CHAPTER-III

## 3 LITERATURE REVIEW

Tuberculosis (TB) is a disease caused by the body's attempt to control the multiplying and spreading of the *M. tuberculosis* (Pace, 2000). Many of the symptoms of TB disease are not caused by the tubercle bacilli themselves but result from immunological hypersensitivity reactions of the host to products of the bacteria. If uncontrolled, these can be destructive to tissues. Thus, TB is a complex of microbiological and immunological events that escapes simple definition (Schlunger *et al*, 1998).

### 3.1 Global History

Tuberculosis is a specific chronic infectious disease caused by *M. tuberculosis* and occasionally by *M. bovis* and *M. africanum*. The disease primarily affects lungs and causes pulmonary tuberculosis. It can also affect intestine, meninges, bone, joints, lymph glands, skin and other tissues of the body. *M. tuberculosis* has been present in the human population since antiquity fragments of the spinal column from Egyptian mummies from 2400 BC; show definite pathological signs of tubercular decay. It has also been referred to in the Vedas and Ayurvedic Samhitas (STC and HIV/AIDS, 2005).

In the past, tuberculosis has been referred to as the "great whit scourge" and by John Bunyan as "the captain of all these men of death". In Ancient Hindu texts, tuberculosis is referred to as Rograj and rajayakshma meaning wasting disease (Sharma *et al*, 2001).

Tuberculosis was well recognized by the time of Hippocrates (377-400 BC), who gave an excellent clinical description of the disease, called "Pthisis", a Greek word which mean "to consume to spit" and "to waste away" (Grange, 1996; Miller, 1982). The

Dutch physician, Franciscus Sylvius (1614-1672) deduced from autopsies that tuberculosis was characterized by the formation of nodules, which he named “tubercles" (Lowell *et al,* 1669).

The transmissible nature of tuberculosis was clearly established by Jean-Antoine Villemin (1827-1892) a French military doctor. In 1868 Villemin published the results of a series of studies in which he convincingly demonstrated that tuberculosis could be produced in rabbits by inoculating them with tuberculous materials from a man or cattle. The disease could be passage from animal to animal and differences in virulence were observed between human and borine material (Lowell *et al,* 1969).

J. L. Schonlein is credited to have named the disease “Tuberculosis” (RossenBlatt, 1973). The word “tuberculosis” which means “a small lamp” (Dubos and Dubos, 1952; Waksman 1964). Several names have been used to refer to tuberculosis in the year gone by; acute progressive tuberculosis has been referred to as; “galloping consumptions”. Pulmonary tuberculosis has been referred as “pulmonali”.

In March 24, 1882, Robert Koch discovered a staining technique that enabled him to see *M. tuberculosis* and announced to discover of the tubercle bacillus and succeeded in culturing it on inspissated serum by a large series of inoculations with pure cultures of the bacillus several generations removed from the primary one. Koch transmitted the disease to many animals of different species. This classical study established without doubt that the bacillus he isolated was the cause of tuberculosis. To this day its demonstration affords the sole infallible criterion for diagnosing tuberculosis in all of its diverse forms (Grange, 1990).

The acid fast nature of the organism was discovered by Ehrlich 1882 (Burke, 1995) and the present method of acid fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence named Ziehl-Neelsen staining technique (Day and Day, 1982).

In 1895, Professor Wilhelm Konard Von Rontgen discovered X-ray and was put to clinical use by 1904. The Finding of radiology and bacteriology helped in developing further knowledge of the disease and correlation between them (Rao, 1981).
In 1904, National Association for the study and prevention of tuberculosis was formed to educate the public and to stimulate programme for the control tuberculosis. In 1920, the International Union Against Tuberculosis was formed (STC and HIV/AIDS, 2005).

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

In 1943, in test animals, Streptomycin, purified form *Streptomyces griseus*, combined maximal inhibition of *M. tuberculosis* with relatively low toxicity. On 20$^{th}$ Nov. 1944, the antibiotic was administered for the first time to a critically ill TB patient. The effect was immediately impressive (STC and HIV/AIDS, 2005).

The major advance in chemotherapy in 1960s was the discovery that therapy could be just as effective when given intermittently (two or three times weekly). As when given daily (TB chemotherapy centre Madras, 1960; Sparbare and Jhnson, 1968; Dayle *et al*, 1979; Stradling and Poole, 1970).

## 3.2 Disease

Tuberculosis is a disease of world wide prevalence and is responsible for more serious illness than any other infectious disease. Despite the availability of effective chemotherapy, it is still a major public health problem in the most countries of the world (Stewart and Beswick, 1977).

The world health organization declared TB a global emergency in 1993, in recognition of its growing importance as a public health problem. TB causes more deaths than any other infectious agent (STC and HIV/AIDS, 2005).

In the last decade, tuberculosis has re-emerged as one of the leading cause of death (nearly 3 million deaths annually) the estimated 8.8 million new cases every year corresponds to 52,000 deaths per week or more than 7,000 each day which translates in to more than 5,000 new cases every day. These death rate however only partially depend the global TB threat, 80% of TB patients are in the economically productive age of 15 to 49 yeas. The emergence of AIDS and decline of socio–economic standard contribute to the disease‘s resurgence in industrialized countries. In most developing countries, although the disease has always been endemic its severity has increased because of the global HIV pandemic and extensive social restructuring due to rapid industrialization and conflicts. The global incidence of TB of the estimated 8.8 million cases world wide more than 40% are in southeast Asia , i.e. WHO has estimated that in three people in the world are infected with TB that compresses 1.7 billion people , 95 % of them living in developing countries (Rablone *et al,* 1995).

TB is a major health problem in the SAARC region causing an immense burden of disease WHO estimates that more than 3 million new TB cases occur in the region with about 1 million deaths every year due to this serious but curable disease. This situation will worsen further with TB/HIV co-infection and MDR TB. The estimated incidence of TB cases for 1995 was 3.095 million in SAARC region which represented about 40% of the global burden of the disease. The incidence rate of all form of TB in 1995 was estimated 41-254 per 1, 00,000 populations (Kumar and Bam, 1996). About 60 % of the adult population have been infected with the TB bacilli in Nepal every year 2,00,000 develops TB and 13,000-15,000 die , are infected patients can transmit the disease to 10-15 people every year and at least 5 % of the newly diagnosed patients with TB have resistance are as more drugs (Sharma and Smith, 1996).

Due to the risk of the spread of disease and the potential for the emergency of drug resistant strains, the diagnosis of the TB is very important, in regards to detection of TB.TB survey conducted in Gorkha demonstrated that there was no gender difference less than 10 year and high prevalence of infection in males than females after this age (Hashimo, 1996). The single most important risk factor for developing tuberculosis is now HIV infection in Nepal. Majority of people with AIDS in the first stage of the epidemics have been commercial sex worker women. The increase occurance of TB cases in woman are due to occurrence of AIDS in female (Smith, 1996).

There are several other reasons for the increase in the incidence of TB, the deterioration of the public health case facilities, the rise in the number of homeless people and people living in congregate settling, the continued influx of immigrants from the countries where TB is endemic and the emergence of patients at risk of TB (Brudney and Dakkin, 1992).

## 3.3 *M. tuberculosis*

*M. tuberculosis* belongs to the family *Mycobacteriaceae*. The Mycobacteria are thin pleomorphic, usually straight or slightly curved rod shaped organisms with parallel sides and round ends, but filamentous and branch forms occur. They are aerobic, non capsulated, non motile and acid and alcohol fast organism (Ananthanarayan and Paniker, 1996; Forbes *et al* 1998).

They vary in width from 0.3 to 6.4 and length from 1-4 micrometer which frequently form small clumps The generic name *Mycobacterium* was proposed by Lehamann and Neumann (1896) in reference to the mould like pellicle formed by MTB on liquid medium (Grange, 1990). The genus *Mycobacterium* is the only genus of the *Mycobacteriaceae* and it is related to other mycolic acid containing genera (Wayne and Kubico, 1986).

The genus has characteristic antigenic pattern and mycolic acid structure. The high G+C content of the DNA of *Mycobacterium* species 66-72 mol % expect for *M. leprae* which has 55 mol % (Grange, 1990).

The size of the *Mycobacterium* depends upon the conditions of growth. In animal tissues they are generally longer and thinner than in cultures. They sometimes show tendency to form filaments in fluid media and old cultures (Rao, 1981; Srivastav and Singhal, 1994).

The bacilli are acid fast, non-motile, non-sporing and have no capsule, arrangement is angular or parallel. Bovine type is more stumpy and stains uniformly, where as, the human type is longer, thinner and curved with granular staining (Day and Day, 1982).

An important character of the Mycobacteria is their ability to resist decolourization by weak mineral acid after being stained by an arylmethane dye-acid fastness, but the genus is more accurately defined by the chemical structure of its mycolic acids and its antigenic structure (Minnikin, 1982).

The cultivable members of the genus are divisible into two major groups the slow growers and rapid growers, which also differ in antigenic structure (Stanford and Grange, 1974) and in DNA relatedness (Baess and Bentzon, 1978).

Electron microscopy shows that Mycobacteria possess a relatively thick cell wall, about 20 nm across. It often appears to be separated from the cell membrane by a thin electron-transparent zone, but this may be an artifact (Imaeda *et al*, 1968).

The cell wall of Mycobacteria is a complex structure of lipid, proteins and polysaccharides. A particular characteristic of Mycobacterial cell wall is the lipid content accounting for 60% of the cell wall weight. Freeze-Fracturing technique reveals that the cell wall has several distinct layers (Barksdale and Kimm, 1977). Cell wall is

made of four distinct layers. Lipids of cell wall particularly the mycolic acid fractions are responsible for acid fastness of bacteria as well as cellular tissue reaction of body. The phosphatide fraction is responsible for tubercle like cellular responses and caseation necrosis.

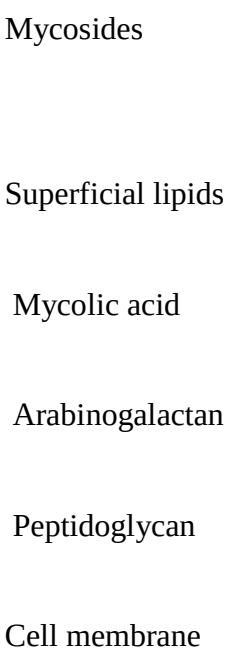



**Figure1** Diagrammatic representation of the *Mycobacterium* cell wall (source Chakraborty 2003)

### 3.3.1 Habitat

*M. tuberculosis* (mammalian) is a strictly parasitic organism. They are usually found in the sputum and faces in case of pulmonary and intestinal tuberculosis and other organs of the body including lymph-nodes, bones, kidneys, meninges. It is also present in dust contaminated with infected sputum (David and Johan 1977; Grange, 1990; Grange and Mointyse, 1979).

There is high frequency of disease in the industrially developed countries, but it has been claimed that immune responsiveness to Mycobacteria is profoundly affected by exposure to them early in life (Stanford and Rook, 1983). Free-living Mycobacteria are usually found in association with watery habitats, marshes, wet soil, streams, Lakes, Rivers and estuaries (Collins *et al,* 1984).

Some species, notably *M. gordonae, M. flavescans and M. marinum* are associated with free water (vialler and viallier 1973). Two species, *M. kansasii and M. xenopi* have colonized water pipes and taps (Bullin *et al,* 1970; Mcswiggan and Collins, 1974).

Mycobacteria also contaminate the taps of containers of distilled water and resins used in deionizers. Hence they may gain access to reagents used for staining and lead in to a number of false reports of small number of acid fast bacilli in sputum. Colonization of ion exchange resin in a renal dialysis machine by *M. chelonei* led to disseminated disease in a patient with renal failure (Azadian *et al,* 1981).

### 3.3.2 Nutritional requirements

The Mycobacteria vary enormously in their metabolic activities, nutritional requirement and rate of growth; these properties are also used for the identification purpose. The basic nutritional requirements of Mycobacteria growing in vitro are carbon, nitrogen, oxygen, phosphorus, sulpher, iron, magnesium and various trace elements. The source of nutrition for the Mycobacteria is probably amino acids and carbohydrates liberated from vegetation by fungal decomposition (Kszada *et al,* 1977).

#### 3.3.2.1 Carbon source

Carbon could be obtained from various source, glycerol is used as a carbon source in most culture media. In the method of gordan and smiath (1953), carbohydrate are

incorporated at concentration of 1% in ammonium phosphate based agar containing bromocresol purple, which serves to detect acid formation. *Mycobacterium* hydrolyses certain glycosides, this property also used for identification purpose (David and Johan 1977; Grange, 1990; Grange and Mointyse, 1979).

### 3.3.2.2 Nitrogen source

Nitrogen is obtainable form inorganic source including ammonium nitrate and form organic nitrogenous compound including wide range of amides, purines, amines, pyrimidine and amino acid. Asparagine and glutamate are particularly useful nitrogen source and are included in most culture media (Grange, 1990).

Variation in nitrogen utilization has been widely used for identification proposes, commonly used test include nitrate reduction and production of ammonia from amides (Bonicke, 1996).

### 3.3.2.3 Source of iron

Mycobactins are water insoluble lipids located in the cell wall. They are synthesized by all cultivable Mycobacteria except *M. paratuberculosis* and some strains of *M. avium*, particularly those from the wood pigeon for this reason; these strains will not grow in vitro unless the medium is supplemented with killed Mycobacteria or pure mycobactin. The mechanism for iron acquisition by these strains in vitro is unknown. Iron is rendered soluble and chelated from the external environment by exochelin and the iron is then transferred to mycobactin for transport across the cell wall. There is evidence that iron complexes to the MS- type exochelin may be transported directly across the cell wall, with the mycobactin serving merely as an iron store (Stapenson and Batledge, 1980).

### 3.3.3 Resistance

*Mycobacterium* are more resistant to drying and chemical disinfectant, temperature 60°C for 20 minutes can kill it, moist heat at 100°C kill it readily. When exposed to sunlight the culture may be killed in 2 hours. In sputum it survives 20 to 30 hours even in sunlight. It is killed by tincture of iodine in 5 minutes and by 80% ethanol in 2 to 10 minutes, Phenol solution 5% kills in 24 hours (Gupte, 1999).

### 3.3.4 Classification

The genus *Mycobacterium* contains 71 recognized or proposed species. These species produce a spectrum of infections in humans and animals ranging from localized lesions to disseminated disease. Many species are also found in water and soil (Grange, 1990). Four very closely related species are responsible for mammalian tuberculosis, *M. tuberculosis* (human tubercle bacillus), *M. bovis* (bovine tubercle bacillus), *M. microti* (vale tubercle bacillus) and *M. africanum* (intermediate in form between human and bovin type).

1) Strict pathogens
*M. tuberculosis* complex
*M. tuberculosis* ------ human type
*M. bovis* ------ bovine type
*M. africanum* ------ human type
*M. microti* ------ murine type

2) leprae bacilli
*M. leprae* ------causing leprosy in man
*M. leprae murium*------causing rat leprosy

3) Other animal pathogens

*M. ulcerans*

*M. balnei*

*M. microti* ------ murine type

*M. para tuberculosis* ------ Johne's bacillus

2) Atypical Mycobacteria

Runyon Group I Photochramogen

Runyon Group II Scotochromogen

Runyon Group III Non- phatochromogen

Runyon Group IV Rapid grower

3) Saprophytic Mycobacteria (non-pathogenic)

*M. smegmatis* ------ present in smegma

*M. phlei* ------ present in grass

*M. stercoris* ------ present in dung

## 3.4 Epidemiology

Tuberculosis is transmitted mainly by droplet infection and droplet nuclei generated by sputum positive patient with pulmonary tuberculosis during coughing, sneezing and vocalizing (Park, 2002). One cough can produce 3,000-5,000 droplet nuclei. Talking, sneezing or singing also produce droplets. They demonstrated that one cough was the equivalent of about 5 minutes of loud taking in terms of the resulting number of droplet nuclei. Tuberculosis is not as easily transmitted as other air borne communicable diseases (Nandell, 1990).

The air droplet nuclei 1-5 micrometers in size are kept suspended by normal air currents. Infection occurs when susceptible person inhales the droplet nuclei (Allen *et*

*al*, 1993). Transmission is by the respiratory route when air borne particle less than 3 micrometer in size inhaled they are not trapped in the nose but may reach the alveoli. One air borne particle (1-3 micrometer) contain 1-10 bacilli. Patient who excrete 10,000 or more tubercle bacilli per ml of sputum are the main source of infection to others (Groothius *et al*, 1991).

Transmission generally occurs indoors where droplet nuclei can stay in the air for long time ventilation removes droplet nuclei. Direct sun light quickly kills tubercle bacilli but they survive in the dark for several hours. Two factors determine an individual's risk of exposure, concentration of droplet nuclei in contaminated air and length of time breathing that air (WHO, 1996).

It is well established that patient with sputum that is positive on direct sputum smear examination are the principle sources of infection (Rouillion *et al*, 1976). The spread of TB in the community may occur by minimal contact with a highly infected person (Rao *et al*, 1980).

### 3.4.1 Incubation period

The time from infection to demonstrable primary lesion or significant tuberculin reaction ranges from 2 -10 weeks while the subsequent risk of progressive pulmonary tuberculosis or extra pulmonary tuberculosis is greatest within the first year or two after infection, latent infection may persist for a lifetime. HIV infection appears to increase the risk greatly and shorten the interval for the development of tuberculosis disease (EDCD, 2000).

### 3.4.2 Risk of infection

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

## 3.5 Predisposing factors in the development of tuberculosis

Predisposing factor for progression of disease are old age, alcoholism, diabetes, neoplastic disease, malnutrition, immunosuppressive drugs, stress and drug induced, congenital or acquired immunodeficiency including HIV infection (Groothius *et al*, 1991). As immunity wanes, through aging or immune suppression, the dormant bacteria reactivate, causing an outbreak of disease often many decades after the initial infection (Cole *et al*, 1998). Immuno-suppression due to diseases of drug therapy is a major predisposing factor for the development of Mycobacterial disease (Groothius *et al*, 1991).

## 3.6 Pathogenesis

The tubercle bacilli owe its virulence to its ability to survive within the macrophage rather than to the production of a toxic substance (Greenwood, 2000).

The essential pathology of tuberculosis consists of the production of a characteristic lesion 'the tubercle'. This is an avascular granuloma composed of a central zone containing giant cells, with or without caseation necrosis surrounded by epithelioid cells and a peripheral zone of lymphocytes and fibroblasts (Ananthanarayan, 1998).

Tubercle bacilli inhibit the fusion of phagosome with lysosome by a mechanism that is poorly understood; the bacilli secrete several compounds (ammonia ions, polyglutamic acid, CAMP and sulpholipids) that affect cell membranes but their role in vivo is uncertain. It has been suggested that the alternation in membrane function may isolate the bacilli from nutrient as well as from the lysosomal contents and this may be the mechanism of bacterial dormancy and persistence (Grange, 1994).

The cell wall of the bacilli induces resistance to infection, causes delayed hypersensitivity, increases reactivity of mice to endotoxin and can replace the whole bacillus freunds's adjuvant. Tubercular-protein can elicit the tuberculin reaction and, when bound to a lipid can induce delayed hypersensitivity. In tissues it induces the formation of monocyte macrophages epiltheloid cells and giant cells. The bacterial polysaccharide induces immediate hypersensitivity and causes exudation of neutrophils from blood vessels into tissues. Lipid causes the accumulation of macrophage and neutrophils phosphatides induce the formation of tubercles consisting of epitheloid cells and giant cells with sometime even caseation (Ananthanarayan, 1998).

### 3.6.1 Primary pulmonary tuberculosis

Primary infection occurs on first exposure to tubercle bacilli. Inhaled droplet nuclei are so small that they avoid the muco-cillary defense of the bronchi and lodge in the terminal alveoli of the lungs. Infection beings with multiplication of tubercle bacilli in the lungs. then form caseous and glaucomatous focal lesion known as Ghon focus. Mycobacteria spread rapidly via the intra pulmonary lymphatic to the regional hilar lymph nodes where the organisms are ingested by the reticuloendothelial cell. Caseous and granulumatous lymphadenitis rapidly ensure causing considerable hilar lymph node enlargement, the primary lung lesion (Gohn Focus) and the regional lymph node lesion are together referred to as the primary complex.

Bacilli may spread in the blood from the vary complex through the body. The immune response delayed hypersensitivity and cellular immunity develops about 4-6 weeks after the primary infection, the size of the infecting dose of bacilli and the strength of the

immune response determine what happen next. In most cases the immune response stops the multiplication of bacilli. However a few dormant bacilli may persist. A positive tuberculin skin test would be the only evidence of infection. The immune response in a few cases is not strong enough to prevent multiplication of bacilli and disease occurs within a few months (WHO, 1996).

### 3.6.2 Post primary tuberculosis

Post primary tuberculosis occurs after a latent period of months or year after primary infection. It may occur either by reactivation of dormant bacilli or by reinfection. Reactivation means that dormant bacilli persisting in tissues for monthly or year after primary infection start to multiply. This may be in response to a trigger, such as weakening of the immune system by HIV infection. Reinfection means a repeat infection in a person who has already previously had a primary infection.

Post primary tuberculosis usually affects the lungs, but can involve any part of the body. The characteristic feature of post primary pulmonary tuberculosis are extensive lung destruction with cavitations, positive sputum smear, upper lobe involvement usually no intrathoracic lymphadenopathy (WHO, 1995).

### 3.6.3 Extra pulmonary TB

Tuberculosis can affect any organ and tissue of the body. The higher rate of infection of extra pulmonary tuberculosis cases in immunocompromised states associated with old age, renal failure, cirrhosis, malnutrition, hematological malignancies and HIV /AIDS. Most, if not all, extra pulmonary lesions results by haematogenous spread of the organism from a primary focus which is not always detected (Chakraborty *et al,* 2001). There are many types of extra pulmonary tuberculosis. These include pleurisy, gland, intestinal, millary, meningitis, bone, urogenital, skin and eye tuberculosis (NTP manual, 1997).

## 3.7 Host defense mechanism

Human displays native immunity to tuberculosis with substantial variation (Hasleton, 1996). The different manifestation of infection with tubercle bacilli reflects the prevalence between the bacilli and host defense mechanism. Traditionally protective immunity to tuberculosis has been described to T–cell mediated immunity with CD4 T cell playing a crucial role. Elimination of *M. tuberculosis* infection mainly depends on the success of the interaction between infected macrophages and T–lymphocytes. CD4 T cell exert their protective effect by the production of cytokines, primarily gamma interferon, after stimulation with mycobacterial antigens. Other T– cell subsets, like CD8 T cells are likely to contribute as well by secreting cytokines and lysing infected cells.

The protective immune response in tuberculosis is by cell mediated immunity. As a result of an effective CMI reaction the primary complex may heal an alternatively the disease may progress in one or more ways (Yates *et al*, 1985).

Macrophages which became activated by lymphokines produced by T lymphocytes can inhibit the intracellular growth of the tubercle bacilli (Schaechter *et al*, 1989). On the other hand, acquired immunity to TB is characterized by the cells after infection or vaccination (Chandrasekar *et al*, 1981). The interplay between *M. tuberculosis* and human host determines the outcome after infection (Cravel *et al*, 2002).

## 3.8 Susceptibility to chemotherapeutic agents

Many chemotherapeutic agents have inhibitory effect on *M. tuberculosis* both in vitro and in vivo but the emergence of strains of tubercle bacilli resistant to one or more drugs has been demonstrated in vitro and in vivo (Tripathy; 1981).

## 3.9 Portal of entry

The MTB infection occurs mainly by inhalation or ingestion but infection may also occur through the skin by a process of inoculation or sometimes through the genito-urinary tact. Direct entry through the conjunctiva is rare. Other portal of entry include

1. Prenatal
2. Trans placental
3. Inhalation
    a) Droplet infection
    b) Dust infection

4. Mouth infection

a) Ingestion

b) Inoculation

5. Genitourinary tract infection

6. Spread of the disease

a) Direct extension

b) Lymphatics

c) Blood stream.

## 3.10 Diagnosis

Rapid and accurate diagnosis of symptomatic patient is a cornerstone of global TB control strategies. The timely identification of person infected with *M. tuberculosis* and their rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measure for the control of positive TB (Noordeen and Godal, 1988).

Diagnosis of active disease includes clinical suspicion – chest radiographs, staining for acid fast bacilli, culture for Mycobacteria and more recently, nucleic acid amplification assay (Foulds and O' Brien, 1998).

### 3.10.1 Clinical diagnosis

The symptoms of PTB in adult include chronic cough with the production of mucopurulant sputum which may contain blood, loss of weight, fever, tiredness, chest pain, anemia and night sweats (during sleep) (Enarson *et al*, 2000; WHO, 1997). The disease in children is not easy to diagnose since there is hardly only productive cough. The symptoms are usually weight loss and enlargement of the lymph glands which may cause obstruction of the bronchi and emphysema, symptoms of extra pulmonary tuberculosis depend on the organ involved (Enarson *et al*, 1996).

### 3.10.2 Laboratory examination

A definitive diagnosis of tuberculosis requires the identification of *M. tuberculosis* bacilli in patient specimen. Conventional procedures for detecting *M. tuberculosis* in specimens usually start with microscopic examination of smear for the presence of acid fast bacilli (Kent and Kubica, 1985).

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

There are several methods of determining the acid fast nature of Mycobacteria in the carbol fuschin (Ziehl- Neelsen) procedure, acid fast organism appear red against a blue back ground, AFB microscopy allows for highly accurate diagnosis to be made by paramedical personal with very little training and using widely available simple and multipurpose equipment (STC Newsletter, 2001).

Nowadays, two methods generally used, the classical Ziehl-Neelsen method and fluorescent method (Hagemann 1938; Lempert, 1944) unfortunately AFB microscopy lacks sensitivity compared with culture. Patient with culture confirmed pulmonary tuberculosis microscopy the sensitivity of AFB microscopy range from 22-80% about 5,000-10,000 AFB/ml of sputum must be present for the detection of TB patient by sputum smear where as culture require only 10-100 viable organism. Hence smear negative culture positive patient overall have minimal disease with low bacillary counts (Colebunders and Bastian, 2000).

#### 3.10.2.1 Staining reaction

Koch (1882) stained the tubercle bacillus with hot alkaline methylene blue as the primary stain and vesuvin as the decolorizer and counter stain. Shortly afterward Ehrliich (1882) discovered the new well known acid fast property; staining the bacilli with hot fuchsin in the presence of aniline oil as a mordant and destaining with a dilute mineral acid. Ziehl changed the mordant to phenol and Neelsen combined the dye and mordant to form carbol fuschisen. Thus the staining technique, although pioneered by Ehrlich, is now known as the Ziehl–Neelsen method (Grange, 1990).

The standard Z-N staining method requires heating the carbol–fuschin covered slide with a flaming torch for several minutes without boiling. Kinyoun′s modification uses a high concentration of carbol fuschin and doesn't require a heating step (Grange, 1990)

The bacilli can be stained without heating if the detergent tergitlal is added to the carbol fuchsin (Srivastava and Singhal, 1994).

The tubercle bacilli can also be stained with carbol auramine on exposure to ultraviolet light, auramine (tetramethyl–diaminodiphenylketomine), a yellow fluorescent dye, fluoresces in the visible region examined with ultraviolet light, tubercle bacilli stained with auramine stain out as bright golden yellow rods against a dark background and are easily identified (Tripathy, 1981). The principle of the staining technique is identical with that of the Ziehl-Neelsen method but without heating (Grange, 1990).

Acid fastness is a property of the intact cell, disintegration of the cell results in loss of acid fastness. Baskesdale and Kim have revised the available information on and have hypothesized that the intact Mycobacterial cell takes carbol fuschin into its intension and also binds fuschin to the mycolic acid residues of the peptidoglycolipids of the outer cell wall. The latter complex once formed, blocks the exist of fuschin, trapped inside. The fuschin inside the cell supplies a brilliant enhancement to the highly staining fuschin–mycolate complex of the outer cell wall. Acid fastness is also lost if the bacilli are exposed to inhibitory action of isonicotinic acid hydrazide (Tripathy , 1981).

**3.10.2.2 Specimen collection and transport**

Acid fast bacilli infect almost any tissue or organ of the body. The successful isolation of the organism depends on the quality of the specimen obtained and the appropriate processing and culture technique employed by the Mycobacterialogy laboratory. Specimens collected in sterile leak proof, disposable and appropriate labeled container and place into bags to contain leakage (Forbes, 1998).

Whenever tuberculosis is suspected, three specimens must be collected for examination by microscopy. The examination of three specimens increases the predictive value of positivity of smear microscopy; reach almost that of culture (Chonde *et al*, 2000).Whenever possible, they should be obtained within twenty four hours (Akhtar *et al*, 2000; WHO, 2000). The overnight specimen is more likely to be positive than the spot specimen. The cumulative positivity is 81%, 93% and 100% for first, second and third sputum respectively (NTP, 2002).

Pulmonary secretion obtained by any of the following methods spontaneously produced or induced sputum, gastric levage, transtracheal aspiration, bronchoscopy and laryngeal swabbing, sputum, aerosol induced sputum, bronchoscopic aspirations and gastric levage constitute the majority of specimens submitted for examination. Spontaneously produced sputum is the specimen of choice. To raise sputum, patients instructed to take a deep breath, hold it momentarily and then cough deeply and vigorously. Patient must also instructed to cover their mouth carefully while coughing and to discard tissues in an appropriate receptacle. Saliva and nasal secretions are not to be collected nor is the patient to use oral antiseptic during the period of collection. Sputum specimen must be free of food particle, residue and other extraneous matter.

The aerosol (saline) induction procedure can best be done ambulatory patients who are able to follow instruction. Aerosol induced sputum have been collected from children as young as specimen delivered promptly to the laboratory and refrigerated if processing is delayed.

### 3.10.2.3 Sputum smear microscopy

The microbiological diagnosis of pulmonary tuberculosis plays a key role in routine and tuberculosis control programmes in developing countries. The cornerstone of the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimen for tubercle bacilli. A direct relationship was demonstrated between positivity in results and mucopurulant sputum. Positivity and bacillary count were found to be lower in these smears (Kusznier *et al*, 2004; Perkins, 2000).

Tuberculosis remains a major public health problem in many parts of the world; microscopic examination of respiratory specimens for AFB plays a key role in the initial diagnosis of tuberculosis, monitoring of treatment and determination of eligibility for release from isolation (Peterson *et al*, 1999)

Beside this, other advantage of AFB microscopy were well known. It is inexpensive to perform and is very specific in high prevalence setting, for these reasons, microscopy rightly stains it's primarily role in case detection as it can detect the most infectious subset of patients (Habeenzu *et al*, 1998; Parekn and Kar, 2003; Selvakumar, 2003). The visualization of AFB in sputum or other clinical materials should be considered only presumptive evidence of TB because stain does not specifically identify *M. tuberculosis* (Forbes *et al*, 1998). It has been observed that between 10,000- 1,00,000 tubercle bacilli per ml of sputum are required for direct microscopy. Sputum specimens from patients with cavitary disease are most often sputum smear positive (Katoach, 2004; Parekh and Kar, 2003).

Microscopic examination of sputum is of great value in making the diagnosis of PTB and in following patient's response to the chemotherapeutic treatment. AFB microscopy allows for highly accurate diagnosis to be made by paramedical personnel with very little training and using widely available simple and multi purpose equipment (STC, 2001). The main values of AFB microscopy for diagnosis lie in its speed extremely high

specificity, while the main disadvantage is said to be its low sensitivity. Specificity is very high, probably over 99% in much high prevalence setting using trained personnel and good microscopes and sensitivity ranges from about 25% to 75%. The minimum number of acid fast bacilli necessary to produce positive smear result has been estimated to be between 5,000 and 10,000 pre milliliter (WHO, 2001). But, microscopy cannot distinguish between live and dead AFB, so that some patients excreting non viable bacilli at the end of treatment may be roughly considered as failure cases (Deun *et al*, 2001).

When all culture positive specimens are considered, the sensitivity of the direct smear compared to that of a smear made from the concentrated specimen was significantly different (Petersen *et al*, 1999). The sensitivity against culture was significantly higher with the concentration method (80%) than with the classic direct smear method (57%) (Garay, 2000). However this concentration method involves incubation at constant temperature, diluting with distilled water and high speed centrifugation. These conditions are not often met by diagnostic facilities in rural areas of developing countries. Petroffs concentration method with NaoH was described in 1968 and is widely used as it increases the sensitivity of sputum smears and also that of culture since the Mycobacteria concentrated in the deposits are viable (Garay, 2000; Perkins, 2000). But when sputum is not adequate i.e. with high number of squamous epithelial cells, the sensitivity of the method is low, many studies reported that concentration methods by the use of NaOCl solution increased the yield of the AFB by more than threefold compared with the direct microscopy of sputum ($P < 0.05$) (Gebre, 2003) . However sediments after concentration with NaCl cannot be used for culture of Mycobacteria. Concentration method also facilitates the examination of the slides and reduces the time required for microscopy (Gebre *et al*, 1997).

Fluorochrome stain is more sensitive than the conventional carbol fuchsin stains because the fluorescent bacilli stand out brightly against the background, the smear can be initially examined at lower magnification (250X – 400X), and therefore more fields

can be visualized in a short period. This makes the method appropriate for use in central or large laboratories with heavy work load but is less feasible in small laboratories because of the associated cost, equipment maintenance and lower specificity (CDC, 2005). The classic carbol fuschsin (ZN) requires heating the slide for better penetration of stain into the Mycobacterial cell wall hence it is also known as the hot stain procedure, Kinyoun acid fast stain is similar to ZN but without heat, hence the term cold stain (Forbes *et al*, 1998).

Singh and Parija (1998) compared the light microscopy of Z-N with that of fluorochrome dye for detection of AFB and reported that out of 2600 clinically suspected patients sputum specimens from 1104 patients were found to be positive for AFB. These included sputum from 975 (37.5 %) patients positive for AFB both ZN and auramine staining methods and sputum from additional 129 (4.96%) patients positive for AFB by auramine staining only.

In a study, Tarhan *et al,* (2003) reported that the sensitivity and specificity of the staining techniques were found as 74% and 88.5% for Auramin flourochrome stain and 86.4% and 96.2% in out of 311 sputum samples for Z-N stain respectively, when culture was accepted as reference method. Ulukanligil *et al,* (2000) found that 68 patients (23.1%) were diagnosed to have TB from 295 patients. The Z-N and fluorochrome microscopy sensitivity were 67.6% (46/68) and 85.2% (58/68) respectively. 201 patients (68.1%) submitted only one specimen to the laboratory. TB positivity was detected in 42 (20.9%) of these patients by culture. However, in 18 patients (6.1%) who submitted two specimens to the laboratory, the TB was positive in 6 (33.3%) of them and Z-N and Auramine fouorochrome microscopy sensitivity were 66% and 83% respectively. In 76 patients who submitted three specimens, TB positivity was determined in 20 (26.3%) of them and the sensitivities were 80% and 92% in the Z-N and Auramine fluorochrome stained smears respectively.

Jain *et al,* (2002) conducted a study to reassess efficiency of the conventional Z-N and Auramine fluorochrome staining techniques of direct microscopy for the detection of AFB from various clinical samples. In the study, a total of 715 consecutive samples comprising 493 sputum samples, 76 cerebrospinal fluid, 66 fine needle aspirates, 30 pus samples and 50 miscellaneous samples were studied and found that 32.3% AFB positivity using both the techniques (Z-N and Auramine stain); 42.2% in sputum and 9.9 in extra pulmonary specimens. Z-N staining showed 23.4% AFB smear positive; 32.7% in sputum and 1.4% in extra pulmonary specimens. Auramine fluorochrome staining showed 31.87% AFB smear positivity, 41.6% in sputum and 9.9% in extra pulmonary specimens. Overall 208 cases were found to be positive in which Z-N contributed only 164 (78.8%) caese which included 3 cases (1.4%) missed by Auramine fluorochrome. The Auramine fluorochrome found 205 cases (98.5%) and missed 3 cases; the difference in cases yields was highly significant ($p<0.001$). In the study Auramine fluorochrome was 86.6% sensitive and Z-N was 67.3% sensitive and a total 46.4% were smear positive while 48.1% were culture positive.

In the study done by Pollock and Wieman (1977), examination of 6,880 sputum specimens from untreated patients disclosed that 3.1% were positive for mycobacteria by Auramine fluorochrome, and 92.5% of these have positive cultures. There was a positive correlation between the number of organisms seen on smear and growth on culture. All specimens contained rare or few AFB on the smear, which was positive smears and negative culture. 82% of the specimens with positive cultures and negative smear yielded less than 25 colonies, whereas there were greater than 25 colonies from 93% of the specimens with positive smears and cultures.

In the study done by Githui *et al,* (1993), a total of 1480 sputum specimens collected from patients with suspected PTB were analyzed. Culture results were used as the gold standard for assessment. Specificity was 97% and 96% for Auramine fluorochrome and Z-N method respectively. The sensitivity of the Auramine fluorochrome method was

80% and that of the Z-N method was 65%. Overall agreement was 86.8%. The use of Auramine fluorochrome greately improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on Z-N stained smears. The method is economical in both time and expense and is recommended for laboratories handling large number of sputum specimens.

Prasanthi and Kumari (2005) also reported that fluorochrome staining was found to be more efficient (45%) when compared to Z-N staining (29%) in detecting cases associated with HIV seropositivity, especially paucibacillary cases. Auromine fluorochrome of auramine stained samples increases sensitivity and saves laboratory time but it is not an appropriate technique for rural areas (Garay, 2000).

Ba and Rieder (1999) found that the yield was similar with both techniques (Z-N and Auromine fluorochrome) for specimens with at least 10 bacilli per 100 fields, but higher yield was found with fluorescence microscopy in those with fewer than 10 bacilli per 100 fields. The mean time required by Auromine fluorochrome before declaring a slide as negative with the same magnification was 3 minutes 34 seconds, compared to 7 minutes 44 seconds with the Z-N technique.

In a study, Tansuphasiri and Kladphurang (2002) evaluated sputum staining by MC method and compared with ZN and fluorochrome method , and study found that out of the 392 sputum sample examined, 22.7%, 19.4% and 22.9% were positive by the ZN, fluorochrome and MC staining methods respectively. In comparison with culture results, the sensitivity of ZN, fluorochrome and MC methods were 68.9%, 59.7% and 70.6% respectively. The results of MC and ZN methods were in close agreement (97.2%). According to Bhat and Bhat (2000) from total 900 specimens screened, 122(13.56%) were found positive by both ZN staining and MC staining, 773 (85.89%) were negative by both methods. In the remaining five samples, 2 were positive by ZN staining and 3 by only cold staining. Kocchar (2002) also observed that the simple MC

staining and simplified concentration is valuable alternation for Mycobacteria in busy clinical laboratories.

Much study reveals that the ability of any screening test to correctly identify diseased patients is directly related to the prevalence of the disease in question. The continuing use of smears for the detection of acid- fast bacilli when the prevalence of TB is declining will produce increasing numbers of false positive results. Data suggest that the smear is a poor screening technique in a population where the prevalence of TB is low (Boyd and Mass, 1975). But Gardin and Slutkin (1900) reported that the positive predictive value of acid fast microscopy was 97.9% and 100% in both low and high laboratory prevalence period of *M. tuberculosis*.

**3.10.2.4 Culture**

Recovery of Mycobacteria from clinical samples consume more time than for normal pathogenic bacteria; requiring as it does the following steps, homogenization, decontamination, centrifugation, neutralization, inoculation of culture media and incubation for up to 8 weeks with regular examination of culture (Mackie and Mc carteney, 1999).

Culture of Mycobacteria requires only 10 to 100 organisms to detect *M. tuberculosis*. As a result, the sensitivity of culture is excellent, ranging from 80% to 93% (ATS-CDC, 2000; Dalouisio *et al*, 1996). Moreover, the specificity is quite high at 98% (ATS- CDC, 2000). Culture increase the sensitivity for diagnosis of *M. tuberculosis* and allow speciation, drug–susceptibility testing and if needed, genotyping for epidemiologic purpose. (ATS-CDC, 2000).

There are three types of culture media**.**

Solid media may be egg based or agar based. This media contains malachite green, a dye that suppresses the growth of contaminating bacteria. Of the egg based media, LJ medium is most commonly used in clinical laboratories. In general LJ medium recovers *M. tuberculosis* well but is not reliable for the recovery of the species. Petragnani medium contains about twice as much malachite green as does LJ medium and is most commonly used for the recovery of Mycobacteria from heavily contaminated specimens. Agar based media are transparent and provide a ready means of detecting early growth of microscopic colonies. Colonies may be observed in 10-12 days in contrast to 18-24 days with opaque egg based media. The distinctive colony characteristics of *M. tuberculosis* are rough, tough and buff colony (Collee *et al*, 1996; Ratton, 2001).

Some medium, such as ogawa's medium, contain egg yolk instead of whole eggs (Grange, 1990).

The addition of glycerol to LJ medium improves the growth of *M. tuberculosis*, but not that of *M. bovis*. Sodium pyruvte, on the other hand increase the growth of *M. bovis* and some strains of drug resistant *M. tuberculosis* (Mackie and Macartney, 1989).

Middlebrook 7H10 and 7H11 media may detect Mycobacteria in less than 4 weeks, but they require incubation for as long as 6 to 8 weeks before they can be classified as negative. (Morgan *et al*, 1983).

*M. tuberculosis* is an obligate aerobe, grows optimally at 37°C (range 25°C to 40°C) and pH 6.4 to 7. It is a slow growing organism with generation time of 14 to 15 hours. The colonies are tenacious and not easily emulsified until recently, identification of *M. tuberculosis* form positive culture depend on biochemical test for niacin, arylsulphatase , neutral red, catalase, peroxidase, amidase and nitrate reductase after the incubation of 2 to 3 weeks ( Forbes *et al*, 2000).

Middlebrook 7H9 and Dubos Tween albumin broths are commonly used for sub culture stock strains of Mycobacteria and preparing inocula for drug susceptibility tests and other in vitro tests (Colee *et al* 1996; Rattan, 2001).

Broth media combined with DNA probes for rapid species identification typically provide result in less than 2 weeks with smear positive samples and somewhat longer with smear negative samples. (Kanchana *et al*, 2000; Morgan *et al*, 1983). Broth media formulation includes both manual and automated systems using radiometric or colorimetric methods for detection of Mycobacteria. Examples of broth media include the BACTEC 460TB and BACTEC MB9000 radiometric methods, the Mycobacterial growth indicator Tube or MGII non–radiometric method and the manual septic-check AFB system (Brodie and Schluner, 2005).

Broth media also may allow more rapid determination of drug susceptibility, particularly if direct susceptibility testing is used. Newer culture technologies are in development. One such product is TK. TK medium uses multi-color dye indicate to identify *M. tuberculosis* rapidly. It can also be used for drug susceptibility testing and can differentiate contaminated specimen (Brodie and Schluner, 2005).

Many specimens, especially sputum, are contaminated by bacteria and fungi; it is necessary to destroy these before culturing for Mycobacteria is made of the ability of Mycobacteria to resist destruction by acid, alkalis and certain disinfectants. Alternatively, specimens may be cultivated in media containing a mixture of antibiotics that inhibit growth of virtually all microorganisms other than Mycobacteria *(M. tuberculosis*). The most widely used these are the 'mitchisen cocktail' containing carebenicillin, trimethoprim, polymyxin B and amphotericin (Mitchison *et al*, 1972; Mitchison *et al*, 1983).

The most popular hard decontamination method is that of petroff (1915), in which sputum or other materials is treated with 4% NaOH for 15 to 30 minutes before being

neutralized some laboratories use 2% NaOH together with the mucolytic agent N-acetyl L-cysteine (Kubica *et al*, 1963). Soft method includes the use of trisodium phosphate (Corper and Stoner, 1943) and acetylpyridinium bromide (Grange, 1990; Makhtari, 1980).

It has been reported that pooled 24 hours sputum collection will yield more positive culture than early morning specimens (Krasnow; 1969) growth is usually slower and the contamination rate is significantly higher in sputum pools (Kestle, 1967). Depending on whether there is minimal or advanced disease, there will be intermittent or continual shedding of tubercle bacilli. A minimum of three and not more than five early morning specimens will usually be sufficient to identify the patient with active disease.

#### 3.10.2.5 Biochemical properties

There is no completely reliable single test that will differentiate *M. tuberculosis* from other Mycobacteria. Therefore, at least one or more precise identification test must be applied besides morphological observation.

Identification tests must be performed with the sub cultured strain, not with the primarily isolated strain. If culture is contaminated, decontamination is required. (Fujiki, 2005).

##### 3.10.2.5.1 Niacin test

This test is based on detection of the presence of nicotinic acid in the culture medium. Nicotinic acid is an intermediate in the biosynthesis of NAD (Nicotin Adenine Dinucleotide). In *M. tuberculosis*, this pathway blocks and dicotinic acid excretes in the culture medium. This test differentiates *M. tuberculosis* (99.5 % species) from most other Mycobacteria.

INH test strips are used for detecting isonicotinic acid (Niacin) and its metabolites in the aqueous extract of organism and medium. The INH test strips are absorbent paper strips impregnated with chloramines T, potassium thiocyanate, citric acid and barbituric acid (Kilburn *et al*). The pyridine ring of isonicotinic acid split by cynogen chloride to form a glutaconaldehyde derivative. The derivative condenses with barbituric acid to form a blue- purple polymethine dye.

**3.10.2.5.2 Nitrate test**

This test based on the principle that the enzyme nitrate reductase causes the reduction of the nitrate, in the presence of a suitable electron donor, to nitrate or nitrogen. This test detects the ability of Mycobacteria to reduce nitrate to nitrite. *M. tuberculosis* and H37RV strain are the nitrate reduction positive where as *M. bovis* and *M. intracellularae* are nitrate reduction negative.

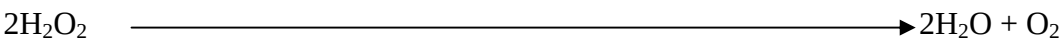
**3.10.2.5.3 Catalase test**

This test is used to differentiate those bacteria that produce the enzyme catalase from non-catalase producing bacteria. Catalase is an intracellular soluble enzyme capable of splitting $H_2O_2$ into water and oxygen. The oxygen bubbles into the reaction mixture indicate catalase activity. Catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria reduced flavoprotein reacts directly with gaseous oxygen to form hydrogen peroxide, which is an oxidative end product of the aerobic breakdown of sugars (Mackie and Mc Carteny, 1999).

$$FPH^2 + O_2 \longrightarrow F^2 + H_2O_2$$

Reduced flavoprotein — oxidized flavoprotein

Hydrogen peroxide thus formed is toxic to bacteria, resulting in their death. The enzyme catalase decomposes hydrogen peroxide in to water and oxygen.




**3.10.2.5.4 Urease test**

The enzyme urease is possessed by some bacteria. The urease is able to decompose urea by hydrolysis to give ammonia and carbon dioxide. The reaction turns the medium alkaline which shows by a change in color of the indicator to red –pink. The indicator is used phenol red (Mackie and Mc Carteny, 1999).


**3.10.2.6 Immunological test**

Use of serum for the detection of different antigens of *M. tuberculosis* can be done by different ELISA technique. Detection of PPD and lipoarabinomannon antigens of *M. tuberculosis* can also be done (Pokharel, 2004). There have recently been significant improvement in serologic tests and in our understanding of the humoral immune response, but the HIV epidemics and its impact on immunity have threatened its progress (Perkins, 2000). Serological technique is not useful in control programmes due to the lack of sensitivity and specificity (Gebre *et al*, 1977).

**3.10.2.7 Antigen detection**

Mycobacterial antigen is detectable in clinical specimens by use of specific antibodies in agglutination technique and enzyme linked immunosorbent assay Elisa development of such test has been overshadowed by DNA technology but result of the few studies of

their use with clean specimens such as cerebrospinal pleural and peritoneal fluids were encouraging with high sensitivities and specificities (wade *et al*, 1990).

#### 3.10.2.8 Antibody detection

The detection of antibodies against Mycobacterial antigens in sera from patients in Elisa has shown promising results the most promising purified antigen in a number of Elisa is the 38 k Da antigen from *M. tuberculosis* the 10 KDa, 16 KDa and 24 KDa proteins isolated from *M. tuberculosis* were also useful in ELISA (Kox, 1996).

##### 3.10.2.8.1 Elispot test

Of the numerous immunological methods for diagnosis of TB one promising test is elispot test it measures the antibody secreting cells in response to stimuli from a specific antigen however owing to its cost and requirements for special expertise this test though useful would not be suitable for use in a routine clinical laboratory (Hoeifetes and Good, 1994).

##### 3.10.2.8.2 Tuberculostearic acid (TBSA) test

One easily detected component of *M. tuberculosis* is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography (Brooks *et al*, 1987). The presence of tuberculostearic acid in cerebrospinal fluid is thought to be diagnostic for tuberculous meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis (Savic *et al*, 1992). However, an important concerns with pulmonary specimens it that organisms other than *M. tuberculosis* may produce components that will generate a false positive signal (Bloom, 1994).

### 3.10.2.9 Molecular method

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

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

Several molecular procedures useful for diagnosis of Mycobacterial disease include strand displacement amplification (SDA), polymerase chain reaction (PCR) amplification, transcription-mediated amplification (TMA), reporter phase systems, oligonucleotide ligation amplification and Q-betaa replicase amplification. The first four of these amplification system are the best developed of the system for Mycobacteria (Bloom, 1994).

#### 3.10.2.9.1 Strand displacement amplification (SDA)

SDA is an isothermal amplification process that takes advantages of ability of DNA polymerase to start at the site of a single-stranded nick in double –stranded DNA, extend one strand from the 3 end, and displace the sown stream strand of DNA (Walker *et al*, 1992). The replicated DNA and the displaced strands are then substrates for additional round of oligonucleotide annealing, nicking and strand displacement such that the amplification proceeds in a geometric manner and can produce $1x10^7$ to$1x10^8$

fold amplification in about 2 hours (Bloom, 1994). The specificity of the SDA reaction is based on the choice of primers to direct the DNA synthesis. When coupled with chemiluminescence- based hybridization detection system, the entire assay can be completed within 4 hours of obtaining a processed specimen (Spargo *et al,* 1993). Species specific SDA assays that have been developed for *M. tuberculosis, M. avium* and *M. kansaii*. An assay that detects many members of the *Mycobacterium* genus (a genus specific assay) has also been developed (Bloom, 1994).

**3.10.2.9.2 Polymerase chain reaction (PCR)**

It is a DNA based technology which produces relatively large number of copies of DNA molecules from minute quantities of source DNA from viable or non viable cell material even when the source DNA is of relatively poor quality (Sambrook *et al*, 1989). PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension (Mullis and Faloona, 1987). Description of numerous PCR-based assays is the detection and identification of individual *Mycobacterium* species, such as *M. tuberculosis, M. leprae* or *M. avium*, has been published recently. In general, the amplification process can be completed in 2 to 4 hours of obtaining a processed specimen and the detection assay can be completed in an additional 2 to 24 hours (Bloom, 1994).

**3.10.2.9.3 Transcription-mediated amplification (TMA)**

TMA, an isothermal target-based amplification system developed by Gen-Probe Incorporation, has been combined with a homogeneous detection method to detect *M. tuberculosis* in clinical specimens (Jonas *et al*, 1993). rRNA is amplified via TMA in which the rRNA target sequences are copied in to a transcription complex by using reverse transcriptase and then RNA polymerase is used to make numerous RNA

transcripts of the target sequence from the transcription complex. The process then repeats automatically. Detection of the amplified sequences is achieved by using an acridinium ester-labeled DNA probe specific for *M. tuberculosis* (Bloom, 1994).

#### 3.10.2.9.4 Reporter mycobacteriophage

A reporter mycobacteriophage is a virus that infects the desired *M. tuberculosis* and produces an easily measured product. The specificity of this approach lies in the host range specificity of the reporter phase. There are phages that can infect only *M. tuberculosis* as well as ones that can grow in several species of Mycobacterium (Jones, 1988). The sensitivity of the system lies in the synthesis of large amount of the reporter product during phase growth (i.e., amplification of the product) and in the sensitivity of the assay in detecting the reporter product. Jacobs *et al* (1993) recently constructed a reporter phage for detecting *M. tuberculosis* that carries the gene for the firefly enzyme luciferase. In the presence of ATP, this enzyme oxidizes luciferin to generate light, which is the reaction that makes firefly glow in the dark (de Wet *et al*, 1987).

Since all these methods require either precision instruments for the amplification or an elaborated method for detection of amplified products due to poor specificity of target sequence selection, which are the major obstacles to wide use of these methods in relatively small scale clinical laboratories such as those in private clinics. In this regards, the loop mediated isothermal amplification (IAMP) has the advantages of rapid reaction , simple operation and easy detection.

### 3.10.2.10 Tuberculin skin test

Historically, the detection of persons infected with *M. tuberculosis* has relied upon procedures that use rather crude preparation of antigens to detect the cell-mediate

immune response to the infecting *Mycobacterium*; the tuberculin or purified protein derivative, skin test (ATS- CDS, 1981).

The earliest preparation of Mycobacterial antigens used experimentally was Koch's tuberculin. The first of these was old tuberculin, a filtrate of old broth culture of tubercle bacilli concentrated by evaporation (Koch, 1891). Later, he in 1901 prepare new tuberculin by finely grinding vaccum-dried tubercle bacilli in a mortar and reconstituting in a glycerol water mixture. In early epidemiological studies with tuberculin, pioneered by clemans van pirquet, non-specific reactions due to constituents of the medium were frequent. Accordingly, Seibert and her colleagues attempted to separate Mycobacterial antigens from impurities in heat concentrated old culture filtrate by precipitating of proteins by 50% ammonium sulphate. The resulting preparation, purified protein derivative (PPD) (Seibert and Glenn, 1941), was and still is used extensively. Even this preparation was for from ideal because the process of autolysis, heating and protein precipitation cause considerable denaturation of the antigens, particularly the species – specific one ( Standford and Rook, 1983).

To avoid denaturation during preparation, standford and his colleagues produced a range of new tuberculin by harvesting Mycobacteria from non-antigenic sauton' medium, washing them, disrupting them in an ultrasonication, separating cytoplasm from cell wall debris by centrifugation, sterilizing the supernatant by repeated membrane filtration and standardizing the protein content of the filtrate. The first of these reagents was burulin, used for studies on *M. ulcercans* infection (Standford *et al*, 1975). Subsequently new tuberculins were prepared from *M. tuberculosis* and a range of environmental Mycobacteria (Standford *et al*, 1983).

Weak or negative tuberculin reactions do not exclude active tuberculosis, some persons are intrinsically non reactive and in other cell mediated immune reactivity is depressed due to advanced age, malnutrition, other concurrent disease or advanced tuberculosis (Rooney *et al*, 1976).

PPD is standardized in terms of its biological reactivity as tuberculin units TU. An international standard is maintained by WHO, one TU is equal to 0.01ml of OT or 0.0002mg PPD (Park and Park, 2004).

The mantoux reaction, obtained by injecting tuberculin into the skin of an individual in whom previous infection with the Mycobacterium has induced a state of cell mediated immunity, is a classic example of delayed hypersensitivity, infection with *M. tuberculosis* sensitizes the person to the antigenic components contained in tuberculin. The process of sensitization takes place mainly in the regional lymph nodes as a result of which sensitized T-lymphocytes enter the blood stream and circulate for long period of time. Their subsequent restimulation with the same or a similar antigen such as an intracutaneous injection of tuberculin elicits a characteristic delayed hypersensitivity reaction with indurations and erythema which peaks at 48 to 72 hours and subsides over a period of 5-6 days.

## 3.11 X-ray

X-ray as a method of screening plays a vital role to identify normal and abnormal shadows. Tuberculosis of the lung can be diagnosed with certain characteristic of chest radiograph. At the same time, this method is equally helpful to differentiate tubercular and non- tubercular disease. Number of studies has proved that radiographic picture (pattern) is absolutely typical of tuberculosis. Many disease of the lung show a similar radiographic appearance and easily imitate tuberculosis. On the other hand, the lesions of pulmonary tuberculosis can take almost any form or radiographic picture, chest radiography can undoubtedly be very helpful in localized abnormalities in the lung (Toman, 1989).

According to Prasai in 1992 criteria of chest radiograph favoring the diagnosis of tuberculosis in NTC.

1. Shadow mainly in the upper zone.

2. Patch or nodular shadow.

3. The presence of a cavity or cavities, although carcinoma or pneumonia may occur in an area of the lung where there is calcification due to tuberculosis.
4. Bilateral shadows, especially if there are in upper zone.

5. The persistence of abnormal shadows without alternation in an x-ray repeated after several weeks of antibiotic treatment.

Pulmonary tuberculosis may mimic many other diseases and difficulty in diagnosis may arise under the following circumstances.

1. Sronchogenic carcinoma.
2. Acute pneumonia and lung abscess.
3. Sarcoidosis.
4. Allergic bronchopulmonary aspergillosis of lung.
5. Extrinsic alveolitis.
6. Pneumococoisis.
7. Gross mediastional lymphoadenopathy associated with post primary pulmonary tuberculosis.

## 3.12 Chemotherapy

The development of effective treatment for tuberculosis has been one of the most significant advances during this century. With the evaluation of controlled trials the chemotherapy of tuberculosis is now more rationally base than in the treatment of other infectious diseases. The objective of treatment is curing i.e. the elimination of both the

fast and slowly multiplying bacilli from the patient's body. The effect of chemotherapy is judged not by the anatomic healing of lesions, but mainly by the elimination of bacilli from the patient's sputum. Chemotherapy should be easily available, free of charge to every patient detected. It should be adequate, appropriate and applied to the entire pool of infections in the community (Park and Park, 2003).

There are new twelve or thirteen drugs active against *M. tuberculosis*, of which six are considered to be essential. An anti-tubercular drug should satisfy the following criteria

1. Highly effective.
2. Free from side effect.
3. Easy to administer.
4. Reasonably cheap.

The currently used drugs may be classified into two groups- Bactericidal and Bacteristatic. The bactericidal drugs kill the bacilli *in vivo*. The bacteriostatic drugs inhibit the multiplication of the bacilli and lead to their destruction by the immune mechanism of the host. (Park and Park, 1991).

Traditionally, anti-tuberculosis drugs have been classified as first line drugs having superior efficacy with acceptable toxicity. These are isoniazide, rifampicin, streptomycin, pyrazinamide, ethmbutol, and second line drugs either having less efficacy , greater toxicity or both. Those are thiacetazone , cycloserine, kanamycin, amikacin, capreomycin, viomycin, para-aminosolichlic acid (Puri and Sachdev, 1994).

# CHAPTER-IV

# 4 MATERIALS AND METHODS

## Materials and chemicals used

A list of materials, chemicals, equipments, biological media and reagents required for this study is given in Appendix.

## Methods

### Study site

This study was conducted at National Tuberculoiss Centre, Thimi, Bhaktapur, Nepal from 29th June 2005 to 29th December 2005.

### Study population

The following two groups of altogether 250x3 sputum samples, three from each patient were included for study:

A) Sputum smear positive by Auramine fluorochrome stain (n=150), one from each patient.

B) Sputum smear negative by Auramine fluorochrome stain (n=100), one from each patient.

**Collection of sputum sample**

Sputum samples were collected easily at any time from patient who expectorates sputum in a large quantity, but from patient with insufficient expectoration an early morning sputum specimen is requested with instruction in how to collect it. The patients who were taking antituberculosis drugs did not need to stop taking the drugs one day before collection, but they must gargle well to remove the food remnants and any antituberculosis drugs remaining in the mouth just before collection.

Three sputum samples from each patient, one on spot and two early morning samples were collected on the consecutive days. The patients were provided with sterile, leak proof, disposable and appropriately labeled wide mouthed container. The patients were instructed to collect 4 to 5 ml deep cough sputum, not saliva nor nasal secretion. Specimens collected should not be exposed to heat and light as tuber bacilli are highly sensitive.

### 4.2.4 Sample evaluation

Sputum is the sample of choice in this study. A good sputum sample consists of recently discharged material from the bronchial tree, with minimum amounts of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume. Ideally, a sputum specimen should have a volume of 3 ml to 5 ml, although smaller quantities are acceptable, if the quality is satisfactory.

When a sputum sample is being collected, adequate safety precaution were taken to prevent the spread of infectious organism. The container was labeled and filled in a request form available in the hospital. The request form contained patients full name, date and address and lab number. The specimen was processed with in 2 hours or if not processed kept at 4°C. The specimen collected was first stained by Auramine fluorochrome stain then Ziehl- Neelsen stain and culture.

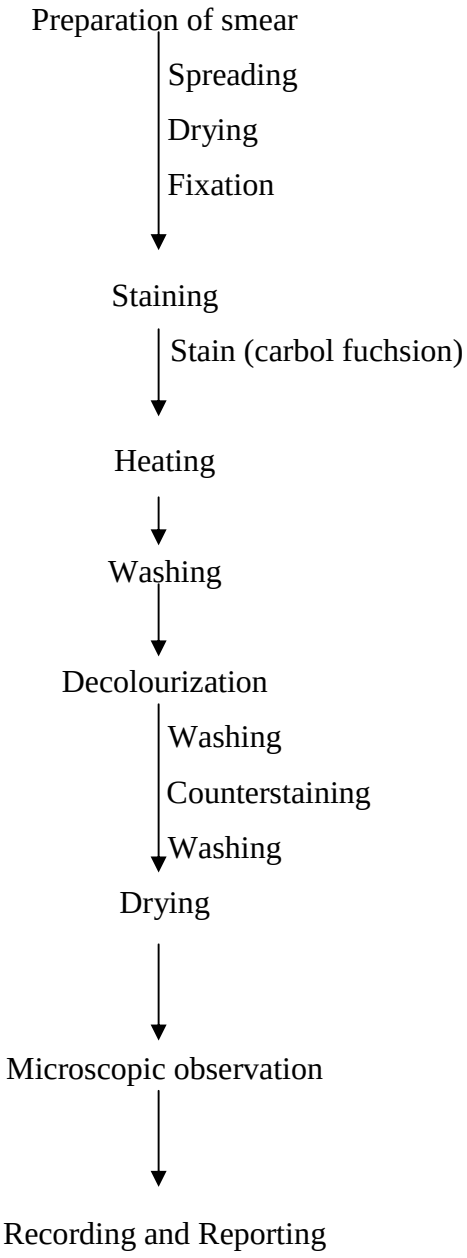



**Figure 2** Flow chart of direct sputum smears examination (Ziehl-Neelsen staining technique)

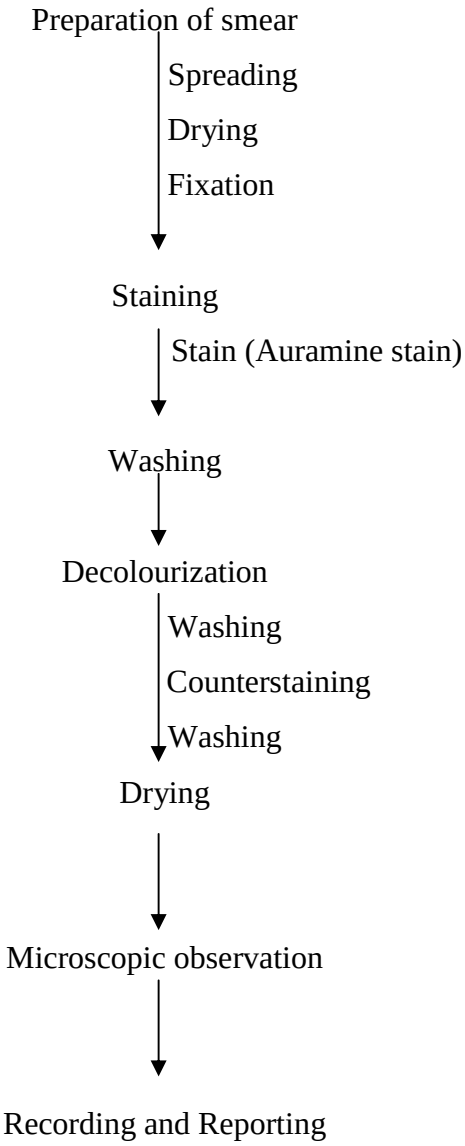



**Figure 2** Flow chart of direct sputum smears examination (Auramine Fluorochrome staining technique)

### 4.2.5 Sputum smear microscopy

#### 4.2.5.1 Ziehl- Neelsen method

**Preparation of smear**

1. The slide was labeled with specimen number and date by diamond pencil.

2. A small portion of sputum (most purulent, most bloody, and most mucoid) was picked.

3. It was spread over the slide in evenly size of about 2x3 cm.

4. Dried it at room temperature completely.

5. It was fixed by passing through the flame 2-3 times, spending for 5 seconds each time without scratching.

**Staining**

1. The heat fixed smear was covered with filtered carbol fuchsin stain.

2. The stain was heated until vapor just began to rise (i.e. about $60^{o}$C).

3. The stain to remain on the slide for 5 minutes.

4. The stain was washed off with clean water.

5. The smear was decolorized by covering the smear with 20% sulphuric acid for 5 minutes or until the smear was sufficiently decolorized i.e. pale pink.

6. The stain was washed off with clean water.

7. The smear was covered with malachite green stain for 1 to 2 minutes.
8. The stain was washed off with clean water.

9. The back of the slide was clean and placed in a draining rack for the smear to air dry.

**Microscopic examination**

1. One drop of oil immersion was put on the left side of the stained smear.

2. The smear was observed first with 40X subjective then with 100 power objective.

3. The examined slide were dipped into xylol to remove the oil immersion at the end of the examination.

**Recording and reporting**

If any definite red bacilli were seen, the smear was reported as AFB positive and reported according to the Bulletin of the International Union Against Tuberculosis IUATLD 1978/ WHO.

More than 10 AFB / field in 20 fields …… (+++).
1 to 10 AFB/ field in 50 fields…… (++).
10 to 99 AFB/ 100 fields …… (+).
1 to 9 AFB/100 fields ……..report the exact number.
No acid fast bacilli found in at least 100 fields…… (-).

#### 4.2.5.2 Auramine fluorochrome method

**Preparation of smear**

1. Sputum specimen number was written in the edge of the glass slide with diamond pencil.

2. A small portion of sputum was picked.

3. About 2-3 cm size smear was prepared.

4. Dried it at room temperature completely.

5. It was fixed passing through the flame 2-3 times, spending about 5 seconds each time.

**Staining**

1. The heat fixed slide was placed on the staining rack, no two slides touching each other.

2. The heat fixed smear of sputum was stained with the auramine solution for 15 minutes at room temperature.

3. The stain was washed off with clean tap water.

4. The slide was covered with 20% sulphuric acid solution for 5 minutes.

5. The slide was washed off with water from clean tap water.

6. The slide was covered with methylene blue solution for 30 seconds.

7. Washed well with water from the tap, allowed to dry in air.

**Microscopic observation**

During microscopic observation, 300 visual fields were examined before reporting as negative, for fluorescent microscopy the light source was switched on for at least 10 minutes before examination is commenced in order to obtain the optimal intensity of illumination.

**Recording and reporting**

ALA scale (American Lung Association in USA)

(--) No acid fast bacilli found.

(-+) 1-2 AFB /300 VF (report the examination of another smear from the same or another specimen.

(+) 3-9 AFB /300 VF.

(++) 10-299 AFB /300 VF.

(+++) more than 300 AFB /300 VF.

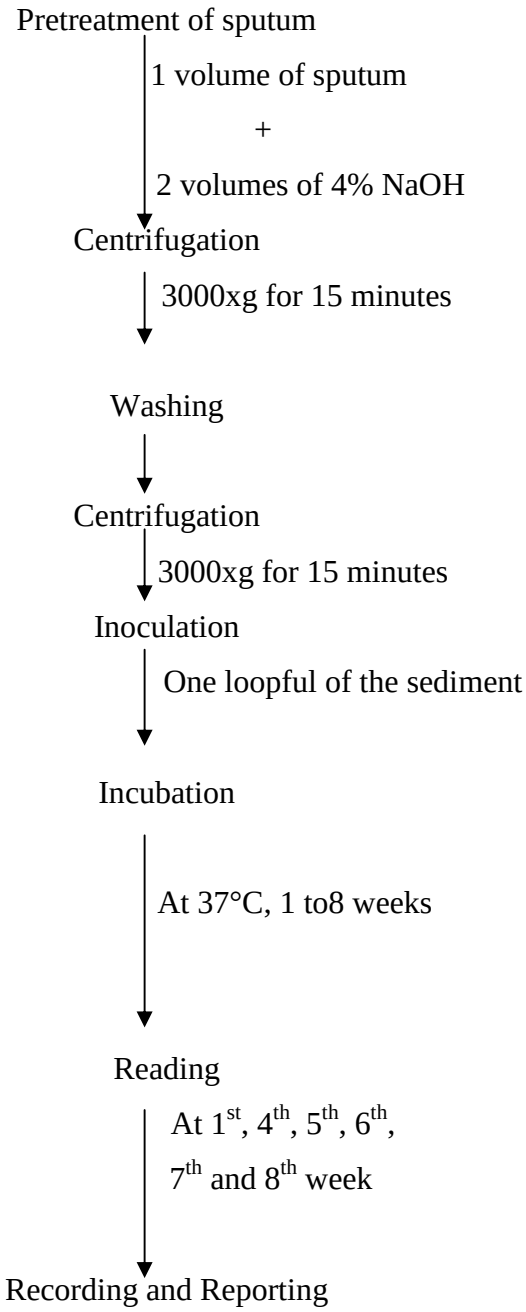



**Figure 4** Flow chart for primary culture examination
(NaOH modified Petroff method)
(Source: Fujiki, Bacteriology Examination to Stop TB)

### 4.2.6 Culture technique (NaOH modified Petroff method)

**Pretreatment**

1. About 2 volumes of 4% NaOH was added to one volume of sputum specimen. Then the cap of the container was tightened and shakes to digest and let stand for 15 minutes at room temperature.

2. The tube was then centrifuged at 3000 x g for 15 minutes then leaved for 10 minutes and poured off the supernatant.

3. 15 ml of sterile distilled water was added and resuspend the sediment.

4. The tubes were centrifuged at 3000 x g for 15 minutes and allowed for 10 minutes and the supernatant were decanted.

5. The condensed water in the medium was removed by putting the media tube upside down on spirit cotton.

6. One drop of sediment was inoculated onto each of two culture tubes.

7. The caps of the inoculated medium was loosened and laid the tubes on the slanting bed and the kept the slants face upward.

**Incubation**

1. The inoculated slants were placed in the incubator at 37°C.

2. The caps were closed tightly when the surface of the media dried; incubation was continued up to at least 8 weeks.

**Observation**

The culture was observed at the 7 day for the rapid growers and at $4^{th}$ week for slow growers.

Growth of colonies on the medium was checked when colonies were observed at each stage (at $7^{th}$ day or $4^{th}$ week); their acid fastness was determined by Z-N staining.

If the colonies do not appear at the time mentioned above, observe weekly until 8 weeks before reported as negative.

**Recording and reporting by WHO**

The growth of AFB and contamination rate was recorded as follows

(--) : no growth.

Actual figure: 1-19 colonies.

(+) :20-100 colonies.

(++) :100-200 colonies.

(+++) :200-500 colonies,almost confluent growth.

(++++) : more than 500 colonies, confluent growth.

**Contamination rate**

C1 + = ¼ of the medium is contaminated.

C2 += ½ of the medium is contaminated.

C3+ = ¾ of the medium is contaminated.

C4 += entire surface of the medium is contaminated.

LQ = the medium is liquefied.

### 4.2.7 Chest X-ray

Chest X-ray was taken by radiographer technologist of NTC and reading of X-ray was done by expert of NTC. In National Tuberculosis Centre, mainly mass miniature X-ray film was taken for tuberculosis of lung, which could be used to diagnose PTB with certain characteristics of chest radiograph. Following criteria of radiography appearance are used for diagnosis of tuberculosis in NTC, shadow in upper zone, nodular shadow, and presence of cavity. Bilateral shadows and the persistence of abnormal shadows without alternation in an X-ray repeated after several weeks of antibiotic treatment.

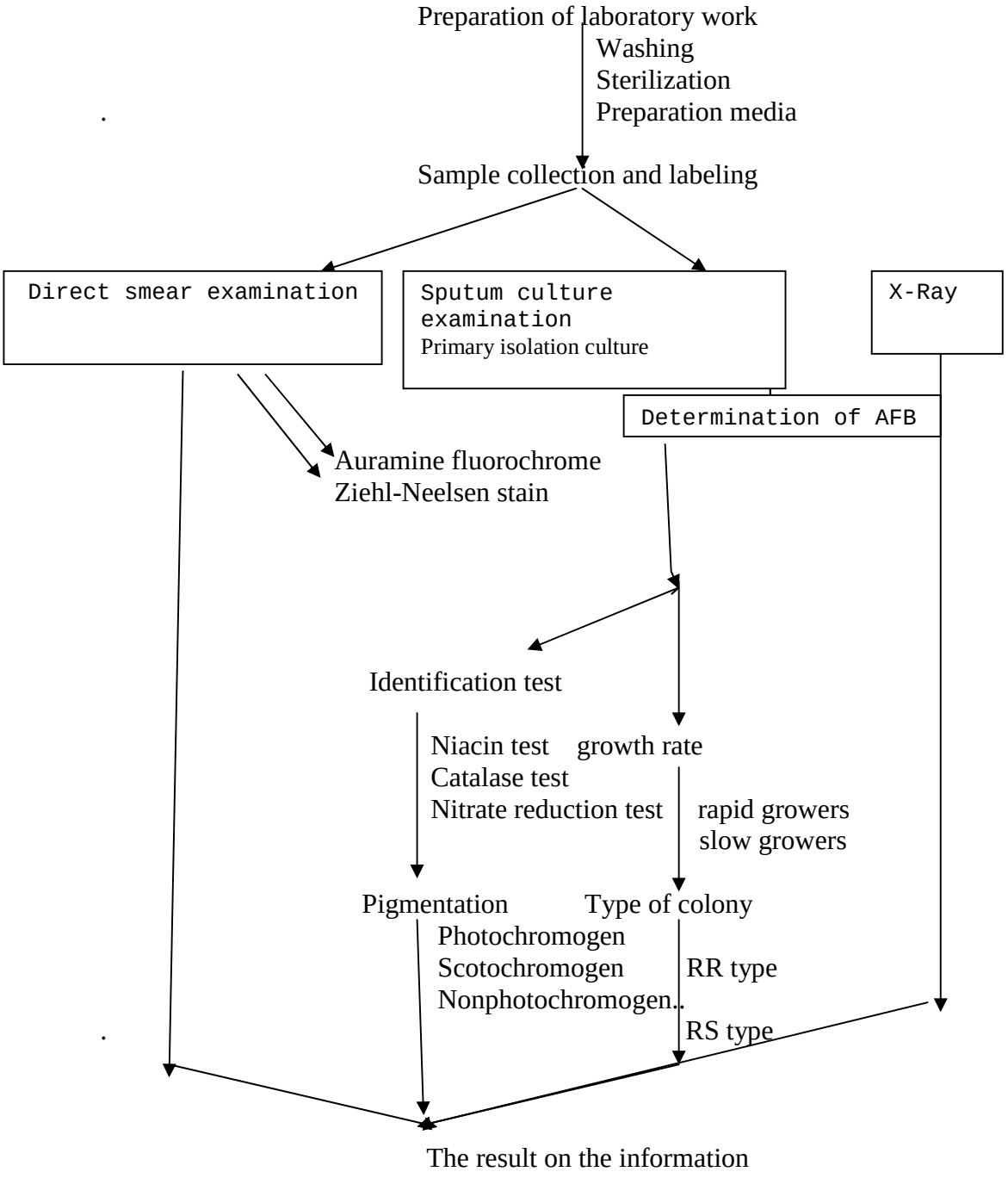



**Figure 5** Flow chart for the examination of TB bacilli
(Source: Fujiki, Bacteriology Examination to Stop TB)

# CHAPTER-V

## 5 RESULTS

The study was conducted among the suspected patients of tuberculosis, attending at the NTC, Thimi, Bhaktapur, Nepal, during 29th June 2005 to December 29th 2005 using different methods. During that study period 250 x 3 samples were taken three each from 250 patients and divided in to two groups A and B. In group A, 150 fluorochrome stain positive samples were taken one each from 150 patient for comparative study of direct sputum smear examination by Ziehl-Neelsen stain, Auramine fluorochrome stain, culture on LJ medium and chest X-ray. Similarly in group B, next 100 fluorochrome stain negative specimens one each from 100 patients were taken for the comparative study of direct sputum smear examination by Ziehl-Neelsen stain, Auramine fluorochrome stain, culture and chest X-ray.

In the study group A (n=150) all the specimens were positive in Auramine fluorochrome stain and all of them show positive in X-ray but only 134 showed positive in Ziehl-Neelsen stain and 136 showed positive in culture.

In the study group B (n=100), all the specimens were negative in Auramine fluorochrome stain and all of them show negative in Ziehl-Neelsen stain but 14 of them were positive in culture and 24 were positive in chest X-ray.

The study was not verified by PCR.

## Study group A

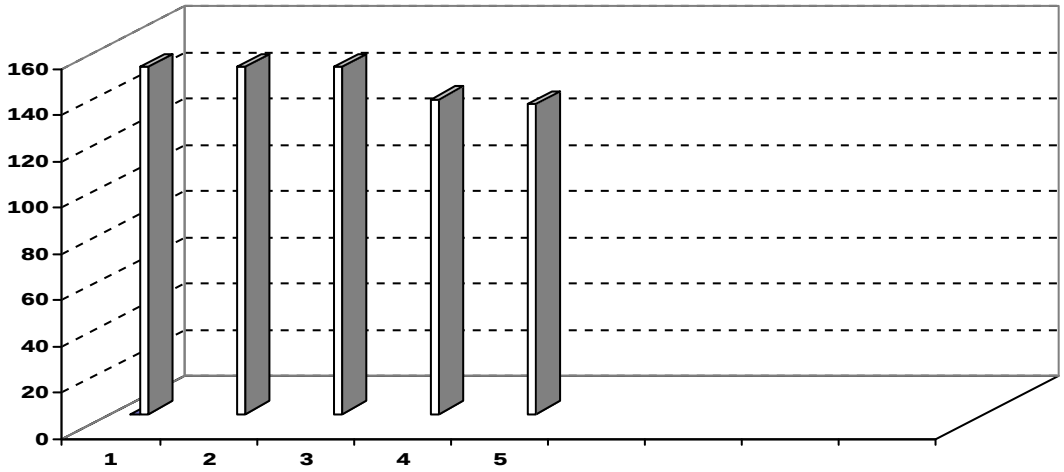



**Figure 6** Comparative study of direct smear examined by Auramine fluorochrome stain, Ziehl-Neelsen stain, culture and chest X-ray

**Table 1** Distribution of positive cases according to different diagnostic method

| S/Bar number | Method | Positive cases | Negative cases |
|---|---|---|---|
| 2 | Fluorescent method | 150 | 00 |
| 3 | X-ray | 150 | 00 |
| 4 | Culture | 136 | 14 |
| 5 | Ziehl-Neelsen method | 134 | 16 |

This group includes 150 direct Auramine fluorochrome smear positive sputum samples, 90.66% of them were positive in cultural examination in LJ medium, 89.33% were positive in Ziehl-Neelsen stain and all the specimens i.e. 100 % were positive in radiological examination.

**Table 2** Distribution of Auramine fluorochrome stain positive cases by age and sex

| S/Bar No. | Age group (year) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| 1 | 0-10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 11-20 | 21 | 14.00 | 3 | 2.00 | 24 | 16.00 |
| 3 | 21-30 | 25 | 16.66 | 4 | 2.66 | 29 | 19.33 |
| 4 | 31-40 | 28 | 18.66 | 8 | 5.33 | 36 | 24.00 |
| 5 | 41-50 | 34 | 22.66 | 6 | 4.00 | 40 | 26.66 |
| 6 | 51-60 | 4 | 2.66 | 3 | 2.00 | 7 | 4.66 |
| 7 | 61-70 | 6 | 4.00 | 2 | 1.33 | 8 | 5.33 |
| 8 | 71-80 | 2 | 1.33 | 1 | 0.66 | 3 | 2.00 |
| 9 | 81-90 | 3 | 2.00 | 0 | 0 | 3 | 2.00 |
| | Total | 123 | 82.0 | 27 | 18.0 | 150 | 100.0 |

Among the studied 150 Auramine fluorochrome stain positive cases, 82% (n=123) were male and 18% (n=27) were female. This study showed that the highest number was seen in the age group 41 to 50 (26.66%), followed by 31 to 40 (24%), 21 to 30 (19.33%), 11 to 20 (16%) and so on.

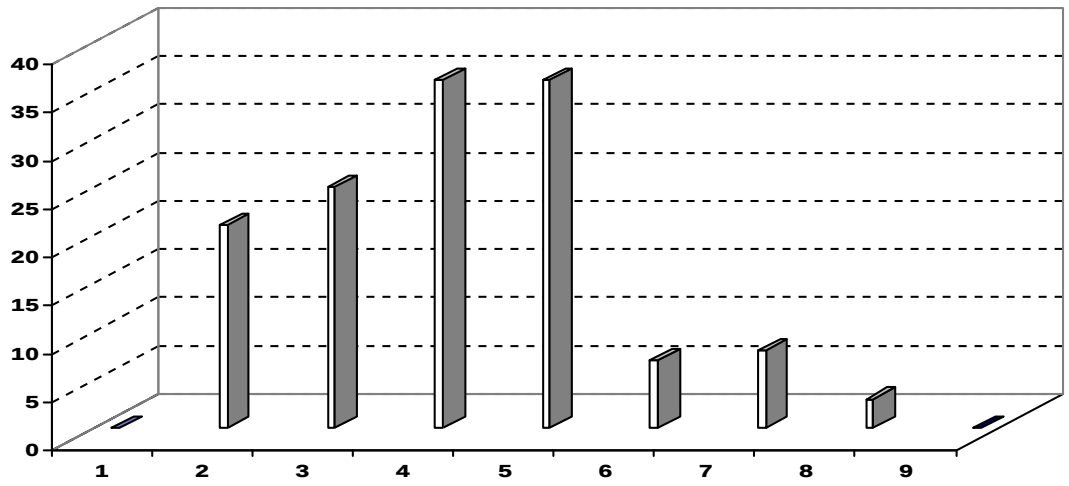



**Figure 7** Distribution of direct smear positive by Auramin fluorochrome microscopy method in different age group

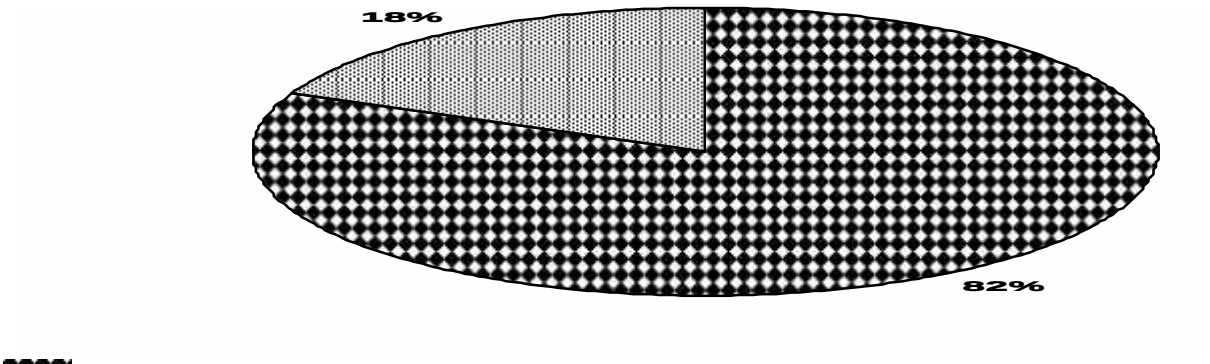



**Figure 8** Prevalence of pulmonary tuberculosis among male and female according to Auramine fluorochrome microscopy method

Out of 150 Auramine fluorochrome positive cases 123 (82%) were male and 27 (18%) were female. This greater occurrence in male than female is statistically significant ($x^2$=61.44) (see calculation No.1).

**Table 3** Distribution of culture positive cases by age and sex

| S/Bar No. | Age group (year) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| 1 | 0-10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 11-20 | 19 | 13.97 | 2 | 1.47 | 21 | 15.57 |
| 3 | 21-30 | 22 | 16.18 | 3 | 2.20 | 25 | 18.38 |
| 4 | 31-40 | 28 | 20.58 | 8 | 5.88 | 36 | 26.47 |
| 5 | 41-50 | 30 | 22.06 | 6 | 4.41 | 36 | 26.47 |
| 6 | 51-60 | 4 | 2.94 | 3 | 2.20 | 7 | 5.15 |
| 7 | 61-70 | 6 | 4.41 | 2 | 1.47 | 8 | 5.88 |
| 8 | 71-80 | 2 | 1.47 | 1 | 0.73 | 3 | 2.20 |
| 9 | 81-90 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Total | 111 | 82 | 25 | 18 | 136 | 100 |

Among the studied 150 Auramine fluorochrome stain positive cases, only 136 cases were positive in culture, in which 82% (n=111) were male and 18% (n=25) were female. This study showed that the highest number was seen in the age group 31 to 40 (26.47%), and 41 to 50 (26.47%), followed by 21 to 30 (18.38%), 11 to 20 (15.57%), 61 to 70 (5.88%) and so on.

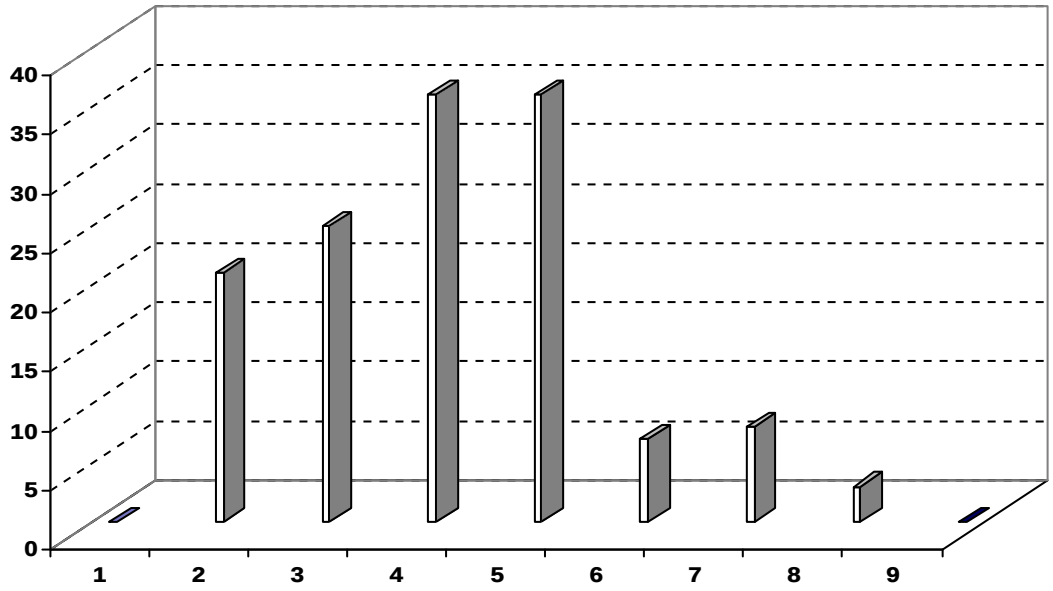



**Figure 9** Distribution of culture positive cases according to different age group

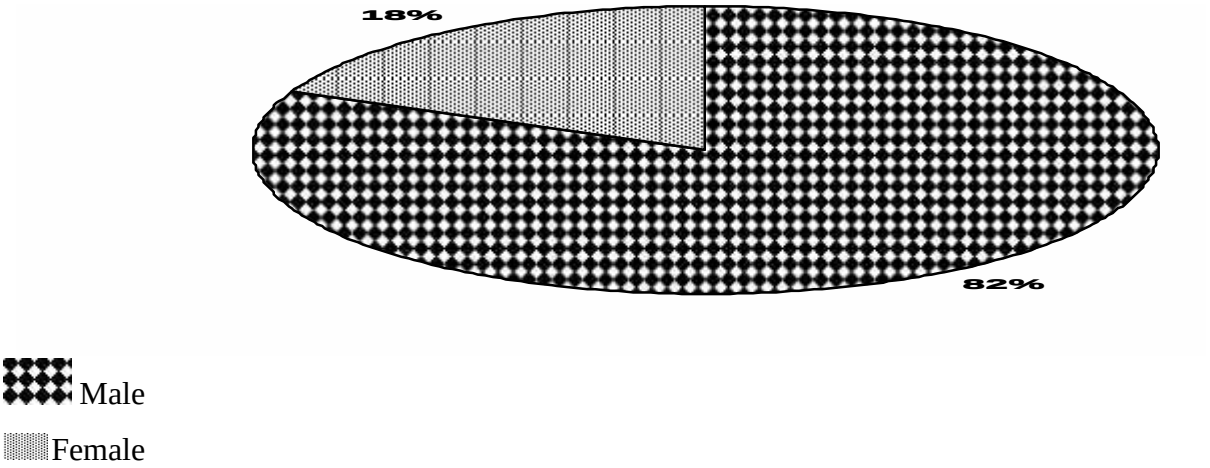



**Figure 10** Prevalence of pulmonary tuberculosis among male and female according to culture.

Out of 136 culture positive cases 111 (82%) were male and only 25 (18%) were female. This greater occurrence in male than female is statistically significant ($x^2$=54.38) (See calculation No.2)

**Table 4** Distribution of chest X-ray positive cases by age and sex

| S/Bar No. | Age group (year) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| 1 | 0-10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 11-20 | 21 | 14.00 | 3 | 2.00 | 24 | 16.00 |
| 3 | 21-30 | 25 | 16.66 | 4 | 2.66 | 29 | 19.33 |
| 4 | 31-40 | 28 | 18.66 | 8 | 5.33 | 36 | 24.00 |
| 5 | 41-50 | 34 | 22.66 | 6 | 4.00 | 40 | 26.66 |
| 6 | 51-60 | 4 | 2.66 | 3 | 2.00 | 7 | 4.66 |
| 7 | 61-70 | 6 | 4.00 | 2 | 1.33 | 8 | 5.33 |
| 8 | 71-80 | 2 | 1.33 | 1 | 0.66 | 3 | 2.00 |
| 9 | 81-90 | 3 | 2.00 | 0 | 0 | 3 | 2.00 |
| 10 | Total | 123 | 82.0 | 27 | 18.0 | 150 | 100.0 |

Among the studied 150 Auramine fluorochrome stain positive cases, all 150 cases were positive in X-Ray, in which 82 % (n=123) were male and 18% (n=27) were female. This study showed that the highest number was seen in the age group 41 to 50 (26.66%), followed by 31 to 40 (24%), 21 to 30 (19.33%), 11 to 20 (16%) and so on.

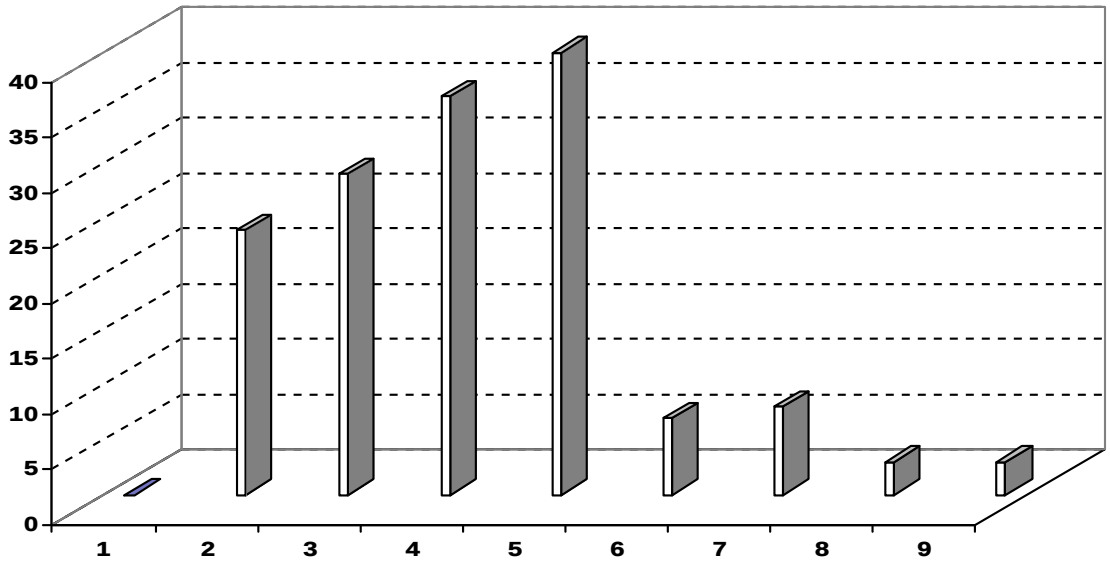



**Figure 11** Distribution of chest X-ray positive cases according to different age group

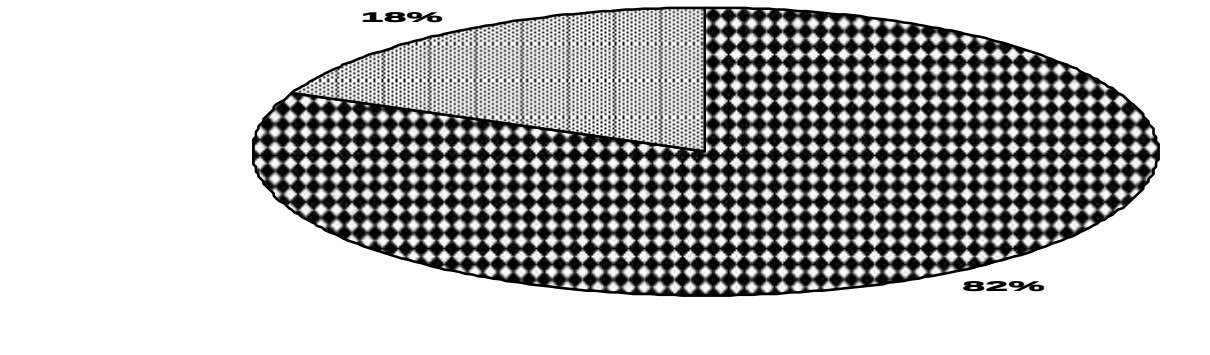



**Figure 12** Prevalence of pulmonary tuberculosis among male and female according to chest X-ray

Out of 150 Auramine Fluorochrome positive cases 123 (80%) were male and 27 (18%) were female. This greater occurrence in male than female is statistically significant ($x^2$=61.44) (see calculation No.3).

**Table 5** Distribution of Ziehl-Neelsen stain positive cases by age and sex

| S/Bar No. | Age group (year) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| 1 | 0-10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 11-20 | 18 | 13.43 | 2 | 1.49 | 20 | 14.92 |
| 3 | 21-30 | 19 | 14.18 | 2 | 1.49 | 21 | 15.67 |
| 4 | 31-40 | 28 | 20.89 | 8 | 5.97 | 36 | 26.86 |
| 5 | 41-50 | 31 | 23.13 | 6 | 4.47 | 37 | 27.61 |
| 6 | 51-60 | 4 | 2.98 | 3 | 2.23 | 7 | 5.22 |
| 7 | 61-70 | 5 | 3.73 | 2 | 1.49 | 7 | 5.22 |
| 8 | 71-80 | 2 | 1.49 | 1 | 0.75 | 3 | 2.24 |
| 9 | 81-90 | 3 | 2.23 | 0 | 0 | 3 | 2.24 |
| 10 | Total | 110 | 82.0 | 24 | 18.0 | 134 | 100.0 |

Among the studied 150 Auramine fluorochrome stain positive cases, all 134 cases were positive in Ziehl-Neelsen stain, in which 82 % (n=123) were male and 18% (n=27) were female. This study showed that the highest number was seen in the age group 41 to 50 (27.61%), followed by 31 to 40 (26.86%), 21 to 30 (15.67%), 11 to 20 (14.92%) and so on.

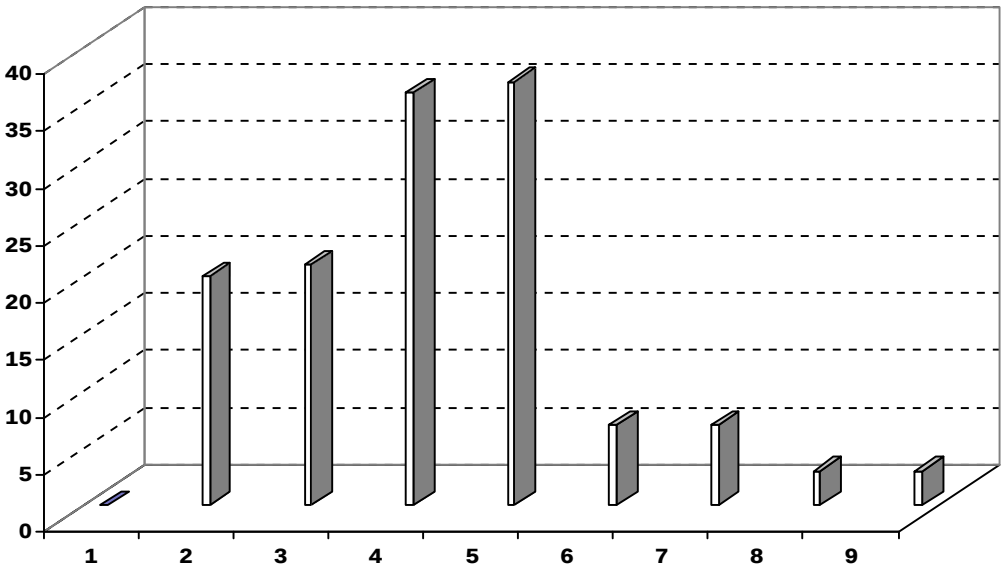
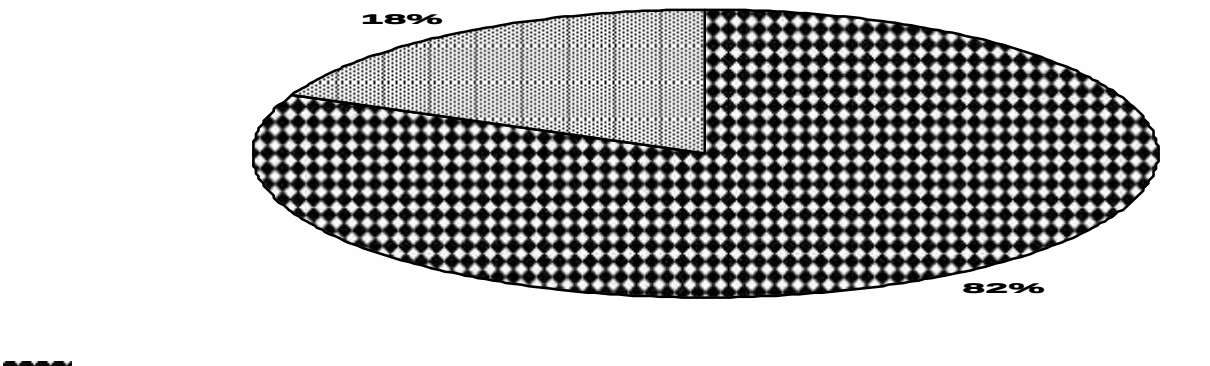



**Figure 13** Distribution of direct smear positive by Ziehl-Neelsen microscopy method in different age group




Male

Female

**Figure 14** Prevalence of pulmonary tuberculosis among male and female according to Ziehl-Neelsen microscopy method

Out of 134 Ziehl-Neelsen stain positive 110 (82%) were male and 24 (18%) were female. This greater occurrence in male than female is statistically significant ($x^2$= 55.18) (See calculation No.4).

## Study group B

In this group, out of 100 Auramine fluorochrome smears negative sputum samples, 24% (24/100) were found to be positive by X-ray and the remaining 76 % (76/100) were negative, where all fluorochrome stain positive samples showed positive in Z-N stain.

Among 100 (100 %) Auramine fluorochrome staining negative sputum samples collected from these cases, 14 % (14/100) samples were positive by culture in LJ medium where as the remaining 86% were negative.

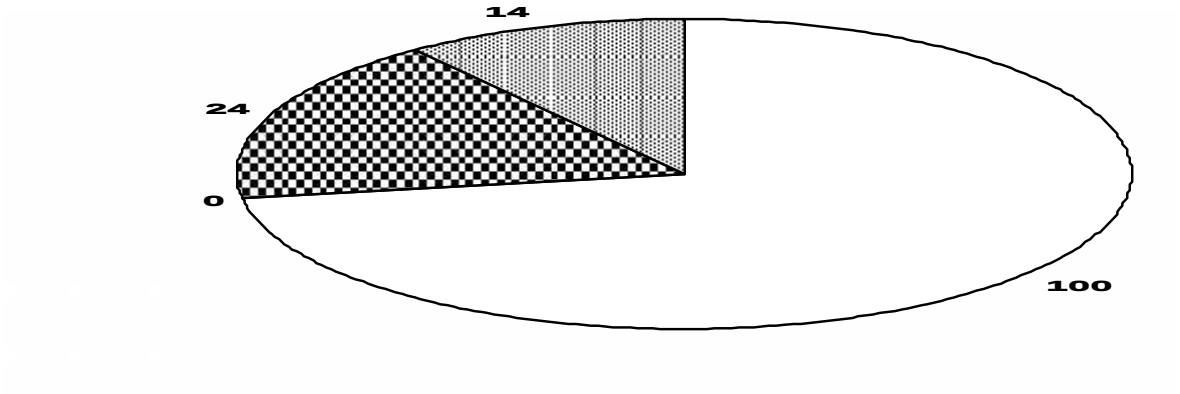



**Figure 15** Comparative study of direct smear examined by fluorescent stain, Ziehl-Neelsen stain, culture and chest X-ray

**Table 6** Distribution of positive cases in direct sputum smears negative cases examined by fluorescent microscopy method

| Methods | Total cases | positive | negative |
|---|---|---|---|
| Fluorescent microscopy | 100 | 000 | 100 |
| Ziehl-Neelsen microscopy | 100 | 000 | 100 |
| X-Ray | 100 | 24 | 76 |
| Culture | 100 | 14 | 86 |

# CHAPTER-VI

## 6 DISCUSSION AND CONCLUSION

### 6.1 Discussion

Direct sputum smear examined either by Auramine fluorochrome stain, Ziehl-Neelsen stain or chest X-ray are the most popular and mainly used method in Nepal for the diagnosis of tuberculosis. *Mycobacterium* culture and anti-tuberculosis drug sensitivity facilities are available only in few hospitals.

According to the WHO guidelines for TB control, patient with more than three weeks history of cough should be screened for pulmonary tuberculosis with direct sputum smear examination for *M. tuberculosis*. Because the clinical signs and symptoms of pulmonary tuberculosis are not specific, the fundamental principal for the diagnosis of tuberculosis is the accurate demonstration of *M. tuberculosis* in a suitable specimen from the suspected cases of PTB for the adequate treatment. Either presumptive diagnosis is based on the demonstration of tubercle bacilli in the sputum or *M. tuberculosis* may be demonstrated by culture. In case of pulmonary tuberculosis, X-ray examinations have been used frequently as a primary screening method (Suitters and Brogger, 1967).

The added advantages of sputum smear microscopy is that it has very close relation with infectiousness, patient who are sputum smear positive and culture positive are for more likely to be infectious than culture positive but smear negative (Narain *et al*,1971).

Although acid fast microscopy is more than hundred years old, it still remains the initial and most rapid step in the diagnosis of tuberculosis, WHO developed new strategies for

global tuberculosis control based on the principal through detecting at least 70% of smear positive cases in the world and curing at least 85% of those detected by the year 2000 (Kochi, 1991).

In this comparative study, the total specimen studied were 250x3, three from each clinically suspected pulmonary tuberculosis patients. These specimens were examined by direct sputum microscopy by Auramine fluorochrome stain, Ziehl-Neelsen stain and Culture. These specimens were divided in two groups A and B. In group A (n=150) one specimen from each patient were taken which all were positive in Auramine fluorochrome stain and compared with chest X-ray, culture and Ziehl-Neelsen stain. Similarly in group B (n=100) one specimen from each patient were taken which all were negative in Auramine fluorochrome stain and compare with chest x-ray, culture and Ziehl-Neelsen stain.

In group A all the specimens were positive in direct sputum microscopy examined by Auramine fluorochrome stain, out of 150 samples only 136 were positive in culture and 134 were positive in direct sputum smear microscopy examined by Z-N stain but all the Auramine fluorochrome stain positive cases showed positive in chest x-ray report, which were read by expert of NTC (National Tuberculosis Center) Thimi, Bhaktapur, Nepal.

In the study group A, 14 patient suspected of PTB were negative in culture for MTB so there may be certain other causes which are responsible for common symptoms to tuberculosis. The reason for negative result in culture may be due to following reasons.

- The patient may have other diseases which share some signs and symptoms of PTB such as systemic mycosis.
- Presence of non-viable bacilli in specimens, as the direct microscopy examinations either Auramine fluorochrome method or Z-N method do not

discriminate between viable and non-viable bacilli, also tubercle bacilli and other Mycobacteria (Gobre *et al*, 1995).

- Inappropriate collection of sputum.
- Fault in transportation of sputum sample.
- Failure in growth of *M. tuberculosis* in media may be due to inappropriate concentration, exposure time of *M. tuberculosis* with alkali during the process of decontamination.
- Misinterpretation of chest X-ray report.
- Use of bactericidal or bacteristatic drugs.
- The media used in this study contained glycerol which inhibits growth Mycobacterium bovis. Therefore, *Mycobacterium bovis* infection shows positive in fluorescent stain do not grow.
- Tubercle bacilli not evenly distributed in sputum specimen.
- Sputum specimen may have expose to sunlight or heat, dried out or contaminated.
- 2 to 3% of AFB positive specimens, both by Auramine fluorochrome and Z-N stain could not be confirmed by growth on LJ medium (Jain *et al*, 2002).

Pulmonary calcification is not always caused by tuberculosis infection. They may arise from other well known conditions, such as certain systemic fungus infection ( Rechert and Campbell, 1952) and parasitic infection.

According to Wetherall, Ledingham and warrell, (1988) there is no absolutely diagnostic radiographic appearance. The following findings support the diagnosis.

- Nodular or patchy shadows in the upper lobes, particularly in the post and apical segments.
- Bilateral upper zone shadows.
- Cavitations in the upper lobes.

- Calcification.
- Linear shadows indicating fibrosis in the upper lobes associated with soft shadows suggesting an active process.
- Mycosis and tuberculosis all produce the same type of pulmonary calcification (Conant *et al*, 1945; Smith, 1947; Edward, 1957).

According to Toman in 1989. The efficacy of chest radiography is determined largely by the reader's ability to detect abnormal opacities and to interpret them correctly. This implies not missing or under reading they are, conversely, not over reading normal opacities on the film. This ability varies from one reader to another. However, it also happens that one and the same reader may at first in an examination of a film see certain abnormalities that he does not see after a week or so, when he examines the same film again. On the other hand, at the second reading, he may find abnormalities on a film that he regarded as normal at the previous examination.

Similar results were obtained by Shrestha German Nepal TB project, Katmandu. Among 1280 PTB suspected cases only 67 cases were sputum smear positive as examined by fluorescent microscopy whereas only 14% of the positive cases were culture positive. Similarly in a study conduced among 100 suspected cases of PTB by chest X-ray, only 82 were sputum smear positive examined by fluorochrome microscopy and 59.7% of the sputum smear positive cases were culture positive (Bhatta, 1996).

In our study, 150 subjects all having the positive in sputum smear examined by fluorochrome stain, only 136 of the sputum smear positive cases were culture positive.

Another study was conducted by Nagpaul (1974) in the National tuberculosis institute, Banglore, India. In this study 2229 outpatients were randomly selected with chest symptoms. 227 were classified by X-ray as tuberculosis but 81 of them were not

confirmed by bacteriological examination. Among the remaining 2002 patients classified as normal or as having a disease other than tuberculosis, 31 of them were found culture positive and smear microscopy. On the other hand among 145 patients positive by smear 130 were confirmed by culture.

Han in 1966 reported that among 1830 patient only 67 were X-ray positive and out of 67 only 13 were positive in culture, this finding were similar to our finding.

In the present study the culture isolates were identified as *M. tuberculosis* observing the following characters.

- Acid fast ness------ present
- Growth rate --------- slow grower
- Type of colony ------ rough, buff and tough.
- Pigmentation --------- non- photochromogens

In this study out of 150 microscopy smear positive by Auramine fluorochroeme stain 90.66 % were culture positive and *M. tuberculosis* is the main causative agent so we could say that *M. tuberculosis* is the main causative agent of tuberculosis among the Nepalese population. MOTT and *M. bovis* were not isolated.

Similarly in study group A among 150 direct sputum smear positive by Auramine fluorochrome stain only 134 were positive in direct sputum smear positive by Z-N method and 16 cases were negative. The reason for negative in Z-N method may be due to following reasons.

- In Z-N method the smear were observed by oil immersion of microscope, more time consuming and it make tedious to the observer.

- Deficiencies in the preparation of the smear such as too little materials spread on the slide or too thin/thick smear.
- Smear prepared from inappropriate sample i.e. from saliva.
- Over heat during fixation and staining procedure.
- Prolong time in decolonization.

Similarly in our study group A among 150 cases of direct sputum smear positive in Auramine fluorochrome stain all cases were positive in chest X-ray examined by expert of the NTC, Thimi, Bhaktapur.

According to Prasai (1992) criteria of chest radiograph favoring the diagnosis of tuberculosis in NTC.

Shadow mainly in the upper zone, Patch or nodular shadow.The presence of a cavity or cavities, although carcinoma or pneumonia may occur in an area of the lung where there is calcification due to TB. Bilateral shadows, especially if there are in upper zone.The persistence of abnormal shadows without alternation in an X-ray repeated after several weeks of antibiotic treatment.

Pulmonary TB may mimic many other diseases and difficulty in diagnosis may arise under the following circumstances.

Bronchogenic carcinoma may be suggested in middle aged smoker by evidence of collapse in the upper lobes or the presence of a solitary round focus. Tomographs showing central cavitations, calcification or the presence of satellite lesions. Although favoring tuberculosis, may also occur in carcinoma. Although favoring tuberculosis may also occur in carcinoma. In the absence of positive sputum conformation of diagnosis may require histological evidence from bronchial biopsy, transbronchial or needle biopsy of the lung or occasionally throractomy. Even the finding of tubercle

bacilli does not exclude carcinoma. Since both diseases may coexist and falsely positive sputum cytology may occur in the presence of active pulmonary tuberculosis.

In acute pneumonia and lung abscess, examination of sputum for acid fast bacilli should not be forgotten, especially if these conditions do not respond to antibiotics chronic pneumonia due to staphylococci, klebsiella pneumonia or anaerobic organisms can result in an illness resembling tuberculosis. The presence of much foul sputum suggests pyogenic abscess.

Sarcoidosis-patients suffering from sarcoidosis typically have few symptoms of tuberculosis and negative in tuberculin tests, confirmations of such patients is done by biopsy of lymph node, kvein test site or lung will establish the diagnosis. It should be remembered that tissue taken from the periphery of caseating tuberculous lesions may show non-caseating granulomata histologically indistinguishable from sarcoidosis.

Allergic bronchopulmonary aspergillosis of lung standing gives rise to fix upper zone shadows resulting from bronchiaectasis and fibrosis. This appearance closely resembling tuberculosis. Patients are usually asthmatic and have characteristic immunological evidence of allergic aspergillosis.

Extrinsic alveolitis, especially avian gives upper lobe shadowing but should be distinguished by a history of exposure to organic antigens and by immunological tests.

Pneumococosis, especially that due to silica or cola may be complicated by tuberculosis and require investigation by careful sputum examination.

The chronic constitutional symptoms associated with pulmonary tuberculosis may be mistaken for those produced by many other disorders, including psychoneurosis, diabetes mellitus and hyperthyroidism and in the elderly may simply be attributed to old

age, tuberculosis should never be forgotten as an important cause of fever of obscure origin.

Gross mediational lymphadenopathy associated with post primary pulmonary tuberculosis is a feature of tuberculosis in immigrants to Britain from Asia and Africa. In distinguishing this from lymphadenopathy due to lymphoma or sarcodosis a strongly positive tuberculin test is help full but lymph-node biopsy by mediastinoscopy may occasionally be necessary (Weatherall *et al,* 1988).

In the present study maximum number of TB cases was observed in the economically most productive age 21 to 50 years, according to the result observed in culture more males were detected i.e. 111 (82%) male and 25 (18%) female among 136 culture positive cases. TB was not diagnosed in the PTB suspects below 10 years. This data shows that the diagnosis of pulmonary tuberculosis in childhood is surrounded by considerably uncertainty. The reason behind this may be that purulent sputum is not available from children .They usually swallow their sputum. The higher occurrence of tuberculosis observed among male than female are due to various reasons they could be the following.

Male have greater exposure to different type of environment or risk factor. Lewis *et al* (1992) have also emphasized on predominance of male over female probably due to lower value ascribed to female in the community, so that they ignore the symptoms of tuberculosis.

Nepal national planning commission (NPC) and UNICEF in 1991 conducted a study in Morang and Sunsary in 125 healthy subjects, Nakanishi and Shrestha 1990 has reported that immunoglobulin M (IgM) and immunoglobulin G (IgG) concentration found in significantly higher in female than in male. The higher concentration of these antibodies in female has protective value hence, low occurrence of tuberculosis among female than male was observed.

Similarly alcohol consumption, khaini and smoking habits were higher in male than female, according to Karki (1993), various study shows that there is positive correlation between alcohol consumption. Khaini and smoking habits and respiratory tract infection. So males were more susceptible than female.

In another study done by Onozaki National TB Programme, 754 patients from the two districts of Dhading and Chitwan were newly registered, among them 454 were new smear positive PTB and 354 were male and 100 were female, the mean age was 36.5 years. This occurrence among male than female and age group is similar to our finding.

Similar type of study was conducted in collaboration with the world health organization in the district of Kailali was to determine the epidemiological situation of tuberculosis. Among 504 persons during the period 1965-72, the diagnosis was confirmed bacteriologically in 379 cases out of 379 cases 58% cases were male and 42% were female. The great majority of new cases was found in middle aged and elderly persons. In our study among 150 persons 136 were positive in culture and all of them were found *M. tuberculosis*, 82% were male and 18% were female. The majority of cases was found in middle age group.

In another study group B among 100 direct sputum smear negative for MTB by Auramine fluorochrome, 14 were culture positive, 24 were X-ray positive and all the cases were also negative in direct sputum smear for MTB by Z-N stain.

In the study, if the validity of test was compared with culture as gold standard. The negative results in direct sputum smear examined by Auramine and Z-N stain may be due to various reasons which may be as follows.

- Inappropriate collection and transport of sputum sample.
- Tubercle bacilli may not have been continuously discharged by patient.
- Tubercle bacilli may not have been evenly distributed in a sputum specimen.

- Early stage of disease.
- Centrifugation of sputum for AFB examination gave mare positive result than un centrifuged sputum. Sputum was not centrifuged which could be the cause of low positive results.
- The sensitivity of the smear method is closely related to the type of pulmonary lesions and the stage at which it is examined. Most frequently, bacilli isolated with cavitory fibrocaseous lesions and fibrocaseous lesions without cavity while in nodular lesion bacilli are isolated in low percentage. Most of the cases in our study may be nodular lesions in lungs. Therefore sputum smear for MTB were found in very low number of positive cases.

## 6.2 Conclusion

The molecular methods like PCR and related techniques are rapid, specific and sensitive. However, these methods require more sophisticated laboratory methods and are not being used for the routine diagnosis of TB. Detection of TB bacilli by sputum smear microscopy is the only feasible method recommended for the Tuberculosis Control Programme in Nepal and many other developing countries in detecting infectious PTB cases and for monitoring the progress of patients during treatment. Since all the Auramine fluorochrome stain positive cases showed positive in X-ray and all the Auramine fluorochrome stain negative cases showed negative in Z-N stain, the Auramine fluorochrome stain positive cases do not require to do X-ray examination and Auramine negative cases do not require to do Z-N stain, it saves time and money but some of the Auramine negative cases showed positive in culture. Hence the study concluded that the diagnosis of PTB could make by Auramine fluorochrome microscopy and culture.

# CHAPTER-VII

## 7 SUMMARY AND RECOMMENDATION

### Summary

Pulmonary tuberculosis is the main public health problem in developing countries. In the present comparative study of direct smear examination for *M. tuberculosis* by Z-N stain, Auramine fluorochrome stain, sputum culture for MTB and chest X-ray, result showed that all the direct sputum smear examination by Auramine fluorochrome stain showed positive in chest X-ray. So it indicates that the direct sputum smear examined by Auramine fluorochrome stain positive do not require chest X-ray. It minimizes the time, expense and labor to treat the PTB patient.

As the PTB suspected patients are treated by the first line of drugs of antituberculosis drugs when the patient is found to positive in direct sputum smear examined by Auramine fluorochrome stain. The major finding of this study is summarized as follows.

In study group A, among 150 direct sputum smear positive examined by auramine fluorochrome stain 123 (82%) were male and 27 (18%) were female.

Among 150 chest x-ray positive cases 123 (82%) were male and 27 (18%) were female.

Among 134 direct sputum smear positive cases examined by Z-N stain, 110 (82%) were male and 24 (18%) were female.

Among 136 sputum culture positive for MTB, 111 (82%) were male and 25 (18%) were female.

In all the positive cases maximum numbers of tuberculosis were observed in the most energetic age group.

Tuberculosis was not diagnosed in suspected cases below 10 years.

## Recommendations

1. In the laboratories handling large number of sputum specimens for tuberculosis diagnosis, Auramine fluorochrome method is recommended.
2. In the staining process, tap water used for washing. Tap water may contain saprophytic acid fast bacilli which contaminate the slide during washing and exhibits AFB in microscopy, so washing of slide and preparation of reagent, distilled water is recommended.
3. Tubercle bacilli may not be continuously discharged by patients, only one sputum smear is not sufficient for identifying tuberculosis cases. Detection of AFB increases with increase in repeated sample number. Hence, examination of three or more early sputum specimen is recommended.
4. Proper diagnosis should be made on the combined basis of different staining technique, chest X-ray and culture.
5. In order to avoid false negative rate and unnecessary burden to patients, molecular techniques like PCR technique should used for diagnosis.
6. Community health education about Tuberculosis must be given.
7. Improve the living standard of people.

# CHAPTER-VIII

## 8 REFERENCES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# APPENDICES

## Appendix I

## Materials

**(A) Bacteriological media**

| | |
|---|---|
| LJ medium | Hi Media laboratories, Pvt Ltd, India |

**(B) Reagents/ chemical**

| | |
|---|---|
| Sulphuric acid | Merck Ltd, Mumbai, India |
| Absolute alcohol | Merck Ltd, Mumbai, India |
| Carbol Fuchsin | S.D fine chem. Ltd, Biosar, India |
| Spirit | Merck Ltd, Mumbai, India |
| Phenol | Merck Ltd, Mumbai, India |
| Methylene blue | Merck Ltd, Mumbai, India |
| Ethanol | Merck Ltd, Mumbai, India |
| Sodium hydroxide | Merck Ltd, Mumbai, India |
| Distilled water | |
| Egg | |
| Glycerol Merck | Ltd, Mumbai, India |
| Malachite green | Merck Ltd, Mumbai, India |
| Cidar wood oil | Hi Media laboratories, Pvt Ltd, India |

**Glasswares**

Beaker

Conical flask

Measuring cylinder

Test tubes

Culture tubes
Glass rods
Pasture pipette
Pipette
Micro pipette
Slides
Petri plate
Glass funnel
Diamond pencil

**Equipments**

| | |
|---|---|
| Microscope | Olympus, Japan |
| Centrifuge | Kuboto, Japan |
| Biological safety cabinet | Kuboto, Japan |
| Autoclave | Kuboto, Japan |
| Incubator | IKemoto, Japan |
| Refrigerator | Sanyo, Japan |
| Coagulator | Hirasawa, Japan |
| Hot air oven | IKemoto, Japan |
| Fluorescent microscope | Olympus, Japan |
| Dryer | Philips |
| Vortex mixer | Remi, equipments, India |
| Electronics balance | Kuboto, Japan |
| Inspissator | chemical and instrumentals corpor, india |

Distilling apparatus
Blender Sanyo, Japan

**Pipettes and tubes**

Eppendrof micropipette

Finnepipette

Micropipette tips

**Miscellaneous**

Bacteriological loop

Cotton

Forceps

Gloves

Staining rack

Spirit lamp

Soaps

Tube holder

Tissue paper

Sputum container

Bamboo stick

Bunsen burner

# Appendix II

# Bacteriological media

**Lowenstein Jensen media**

Preparation of the International Union against Tuberculosis Lowenstein Jensen medium (IUTM) (IUTLD-1998).

**IUTM base composition**

| Ingredients | Composition |
|---|---|
| L- Asparagine | 2.25gm |
| Potassium phosphate, dibasic, anhydrous | 1.50gm |
| Magnesium sulphate 7H2O | 0.4gm |
| Magnesium citrate | 0.6gm |
| Malachite green | 0.25gm |

(A) Preparation of Lowenstein Jensen medium

| Ingredients | Composition |
|---|---|
| IUTM base | 31.0gm |
| Glycerol | 10 ml |
| Distilled water | 500 ml |

- Thirty one gram of IUTM base was Mix well in 500 ml of distilled water and heat 100°C for 30 minutes in a water bath (or autoclave at 121°C for 15 minutes).

- Add glycerol 10 ml into the salt solution.

(B) Preparation of whole egg homogenate

- Wipe off egg shell with spirit cotton.
- Break down the egg in to a Petri plate to check the decomposition.
- Transfer the egg into the beaker (500ml).
- Homogenize the egg with the help of blender until the egg became watery.
- Place the two layer of sterile gauze piece on the funnel.
- Filter the egg homogenate till 830 ml.

(C) Mix A with B (raw medium).

**(C)** Distribution of raw medium.

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

**(D)** Inspissations

Arrange the tubes in slant position and coagulate them at 90°C for one hour with caps closed loosed.

(F) Store at 4°C to 6°C with caps closed tightly.

## Appendix III

## Reagents/chemicals

### 1) Reagents for Auramine fluorochrome staining

A. For Auramine fluorochrome solution

| | |
|---|---|
| Auramine | 1 gm |
| Phenol | 30 ml |
| Distilled water | 870 ml |
| Ethanol (90%) | 100 ml |

B. 20% Sulphuric acid

| | |
|---|---|
| Sulphuric acid | 200 ml |
| Distilled water | 1000 ml |

C. 0.1% Methylene blue

| | |
|---|---|
| Methylene blue powder | 1 gm |
| Distilled water | 1000 ml |

### 2) Reagents for Ziehl –Neelsen staining

A. Ziehl-Neelsen carbol fuchsin

| | |
|---|---|
| Basic fuchsin | 10 gm |
| Ethanol or methanol, absolute | 100 ml |
| Phenol | 50 gm |
| Distilled water | 1000 ml |

B. 0.5% Malachite green

| | |
|---|---|
| Malachite green | 5 gm |

Distilled water 1000 ml

## Appendix IV

## Master chart

C) Study group A Sputum smear positive by Auramine fluorochrome stain (n=150), one from each patient.

| S. No | Age | Sex | Ziehl-Neelsen | Fluorochrome | Culture | X-Ray |
|---|---|---|---|---|---|---|
| 1 | 19 | Male | Positive | Positive | Positive | Positive |
| 2 | 28 | Male | Positive | Positive | Positive | Positive |
| 3 | 44 | Female | Positive | Positive | Positive | Positive |
| 4 | 53 | Male | Positive | Positive | Positive | Positive |
| 5 | 16 | Male | Positive | Positive | Positive | Positive |
| 6 | 44 | Male | Positive | Positive | Positive | Positive |
| 7 | 50 | Male | Positive | Positive | Positive | Positive |
| 8 | 25 | Male | Positive | Positive | Positive | Positive |
| 9 | 36 | Male | Positive | Positive | Positive | Positive |
| 10 | 52 | Female | Positive | Positive | Positive | Positive |
| 11 | 32 | Male | Positive | Positive | Positive | Positive |
| 12 | 20 | Male | Positive | Positive | Positive | Positive |
| 13 | 18 | Female | Negative | Positive | Negative | Positive |
| 14 | 31 | Female | Positive | Positive | Positive | Positive |
| 15 | 47 | Male | Positive | Positive | Positive | Positive |
| 16 | 29 | Male | Positive | Positive | Positive | Positive |
| 17 | 14 | Male | Positive | Positive | Positive | Positive |
| 18 | 63 | Male | Positive | Positive | Positive | Positive |
| 19 | 87 | Male | Positive | Positive | Negative | Positive |
| 20 | 35 | Female | Positive | Positive | Positive | Positive |
| 21 | 27 | Male | Negative | Positive | Positive | Positive |
| 22 | 48 | Male | Positive | Positive | Positive | Positive |
| 23 | 17 | Male | Positive | Positive | Positive | Positive |
| 24 | 30 | Male | Positive | Positive | Positive | Positive |
| 25 | 49 | Male | Positive | Positive | Positive | Positive |
| 26 | 31 | Male | Positive | Positive | Positive | Positive |
| 27 | 42 | Male | Positive | Positive | Positive | Positive |
| 28 | 27 | Male | Positive | Positive | Positive | Positive |
| 29 | 17 | Male | Positive | Positive | Positive | Positive |
| 30 | 23 | Male | Positive | Positive | Positive | Positive |
| 31 | 47 | Male | Positive | Positive | Positive | Positive |
| 32 | 32 | Male | Positive | Positive | Positive | Positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 33 | 57 | Male | Positive | Positive | Positive | Positive |
| Contd. | | | | | | |
| 34 | 49 | Female | Positive | Positive | Positive | Positive |
| 35 | 48 | Male | Positive | Positive | Positive | Positive |
| 36 | 33 | Male | Positive | Positive | Positive | Positive |
| 37 | 27 | Male | Positive | Positive | Positive | Positive |
| 38 | 77 | Female | Positive | Positive | Positive | Positive |
| 39 | 15 | Male | Positive | Positive | Positive | Positive |
| 40 | 21 | Male | Positive | Positive | Positive | Positive |
| 41 | 56 | Female | Positive | Positive | Positive | Positive |
| 42 | 20 | Male | Positive | Positive | Positive | Positive |
| 43 | 39 | Male | Positive | Positive | Positive | Positive |
| 44 | 50 | Female | Positive | Positive | Positive | Positive |
| 45 | 31 | Male | Positive | Positive | Positive | Positive |
| 46 | 25 | Male | Positive | Positive | Positive | Positive |
| 47 | 35 | Male | Positive | Positive | Positive | Positive |
| 48 | 18 | Male | Positive | Positive | Positive | Positive |
| 49 | 14 | Male | Positive | Positive | Positive | Positive |
| 50 | 24 | Male | Positive | Positive | Positive | Positive |
| 51 | 34 | Male | Positive | Positive | Positive | Positive |
| 52 | 40 | Female | Positive | Positive | Positive | Positive |
| 53 | 49 | Male | Positive | Positive | Positive | Positive |
| 54 | 33 | Male | Positive | Positive | Positive | Positive |
| 55 | 64 | Male | Positive | Positive | Positive | Positive |
| 56 | 16 | Male | Positive | Positive | Positive | Positive |
| 57 | 26 | Male | Positive | Positive | Positive | Positive |
| 58 | 34 | Male | Positive | Positive | Positive | Positive |
| 59 | 21 | Male | Positive | Positive | Positive | Positive |
| 60 | 34 | Male | Positive | Positive | Positive | Positive |
| 61 | 43 | Female | Positive | Positive | Positive | Positive |
| 62 | 46 | Male | Positive | Positive | Positive | Positive |
| 63 | 31 | Male | Positive | Positive | Positive | Positive |
| 64 | 15 | Male | Positive | Positive | Positive | Positive |
| 65 | 28 | Male | Positive | Positive | Positive | Positive |
| 66 | 65 | Male | Positive | Positive | Positive | Positive |
| 67 | 16 | Male | Positive | Positive | Positive | Positive |
| 68 | 33 | Male | Positive | Positive | Positive | Positive |
| 69 | 79 | Male | Positive | Positive | Positive | Positive |
| 70 | 34 | Female | Positive | Positive | Positive | Positive |
| 71 | 16 | Male | Negative | Positive | Positive | Positive |
| 72 | 47 | Male | Positive | Positive | Positive | Positive |
| 73 | 12 | Male | Positive | Positive | Positive | Positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 74 | 29 | Male | Positive | Positive | Positive | Positive |
| Contd. | | | | | | |
| 75 | 19 | Male | Positive | Positive | Positive | Positive |
| 76 | 59 | Male | Positive | Positive | Positive | Positive |
| 77 | 47 | Male | Positive | Positive | Positive | Positive |
| 78 | (35) | Male | Positive | Positive | Positive | Positive |
| 79 | 44 | Female | Positive | Positive | Positive | Positive |
| 80 | 20 | Male | Positive | Positive | Positive | Positive |
| 81 | 30 | Male | Positive | Positive | Positive | Positive |
| 82 | 37 | Male | Positive | Positive | Positive | Positive |
| 83 | 43 | Male | Positive | Positive | Positive | Positive |
| 84 | 36 | Male | Positive | Positive | Positive | Positive |
| 85 | 44 | Male | Positive | Positive | Positive | Positive |
| 86 | 14 | Male | Positive | Positive | Positive | Positive |
| 87 | 49 | Male | Positive | Positive | Positive | Positive |
| 88 | 24 | Male | Positive | Positive | Positive | Positive |
| 89 | 44 | Male | Positive | Positive | Positive | Positive |
| 90 | 29 | Female | Positive | Positive | Positive | Positive |
| 91 | 33 | Male | Positive | Positive | Positive | Positive |
| 92 | 42 | Male | Positive | Positive | Positive | Positive |
| 93 | 81 | Male | Positive | Positive | Negative | Positive |
| 94 | 41 | Male | Positive | Positive | Positive | Positive |
| 95 | 16 | Male | Positive | Positive | Positive | Positive |
| 96 | 23 | Male | Positive | Positive | Positive | Positive |
| 97 | 56 | Female | Positive | Positive | Positive | Positive |
| 98 | 19 | Female | Positive | Positive | Positive | Positive |
| 99 | 23 | Female | Negative | Positive | Positive | Positive |
| 100 | 39 | Male | Positive | Positive | Positive | Positive |
| 101 | 43 | Male | Positive | Positive | Positive | Positive |
| 102 | 69 | Male | Positive | Positive | Positive | Positive |
| 103 | 21 | Male | Positive | Positive | Positive | Positive |
| 104 | 12 | Male | Negative | Positive | Negative | Positive |
| 105 | 44 | Male | Positive | Positive | Positive | Positive |
| 106 | 34 | Female | Positive | Positive | Positive | Positive |
| 107 | 42 | Male | Positive | Positive | Positive | Positive |
| 108 | 33 | Male | Positive | Positive | Positive | Positive |
| 109 | 23 | Male | Positive | Positive | Positive | Positive |
| 110 | 47 | Male | Positive | Positive | Positive | Positive |
| 111 | 35 | Male | Positive | Positive | Positive | Positive |
| 112 | 38 | Female | Positive | Positive | Positive | Positive |
| 113 | 60 | Male | Positive | Positive | Positive | Positive |
| 114 | 39 | Male | Positive | Positive | Positive | Positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 115 | 37 | Male | Positive | Positive | Positive | Positive |
| Contd. | | | | | | |
| 116 | 70 | Male | Positive | Positive | Positive | Positive |
| 117 | 48 | Male | Positive | Positive | Positive | Positive |
| 118 | 69 | Female | Positive | Positive | Positive | Positive |
| 119 | 24 | Male | Negative | Positive | Positive | Positive |
| 120 | 40 | Female | Positive | Positive | Positive | Positive |
| 121 | 72 | Male | Positive | Positive | Positive | Positive |
| 122 | 24 | Male | Negative | Positive | Negative | Positive |
| 123 | 38 | Male | Positive | Positive | Positive | Positive |
| 124 | 26 | Male | Negative | Positive | Negative | Positive |
| 125 | 45 | Female | Positive | Positive | Positive | Positive |
| 126 | 29 | Male | Negative | Positive | Negative | Positive |
| 127 | 37 | Male | Positive | Positive | Positive | Positive |
| 128 | 26 | Female | Negative | Positive | Positive | Positive |
| 129 | 44 | Male | Positive | Positive | Positive | Positive |
| 130 | 46 | Male | Positive | Positive | Positive | Positive |
| 131 | 44 | Male | Negative | Positive | Negative | Positive |
| 132 | 40 | Male | Positive | Positive | Positive | Positive |
| 133 | 49 | Male | Positive | Positive | Negative | Positive |
| 134 | 19 | Male | Negative | Positive | Negative | Positive |
| 135 | 25 | Female | Positive | Positive | Positive | Positive |
| 136 | 47 | Male | Positive | Positive | Positive | Positive |
| 137 | 48 | Male | Negative | Positive | Negative | Positive |
| 138 | 43 | Male | Positive | Positive | Positive | Positive |
| 139 | 46 | Male | Negative | Positive | Negative | Positive |
| 140 | 36 | Female | Positive | Positive | Positive | Positive |
| 141 | 42 | Male | Positive | Positive | Positive | Positive |
| 142 | 44 | Male | Positive | Positive | Positive | Positive |
| 143 | 84 | Male | Positive | Positive | Negative | Positive |
| 144 | 38 | Male | Positive | Positive | Positive | Positive |
| 145 | 65 | Male | Negative | Positive | Positive | Positive |
| 146 | 41 | Male | Positive | Positive | Positive | Positive |
| 147 | 65 | Female | Positive | Positive | Positive | Positive |
| 148 | 39 | Male | Positive | Positive | Positive | Positive |
| 149 | 14 | Female | Positive | Positive | Positive | Positive |
| 150 | 25 | Male | Negative | Positive | Negative | Positive |

D) Study group B Sputum smear negative by Auramine fluorochrome stain (n=100), one from each patient.

| S. No | Age | Sex | Fluorochrome | Ziehl- Neelsen | Culture | X-Ray |
|---|---|---|---|---|---|---|
| 1 | 14 | Male | Negative | Negative | Negative | Negative |
| 2 | 20 | Male | Negative | Negative | Positive | Positive |
| 3 | 54 | Female | Negative | Negative | Negative | Negative |
| 4 | 63 | Male | Negative | Negative | Negative | Negative |
| 5 | 16 | Male | Negative | Negative | Negative | Negative |
| 6 | 44 | Male | Negative | Negative | Negative | Negative |
| 7 | 80 | Male | Negative | Negative | Positive | Positive |
| 8 | 25 | Male | Negative | Negative | Negative | Negative |
| 9 | 76 | Male | Negative | Negative | Negative | Positive |
| 10 | 52 | Female | Negative | Negative | Negative | Negative |
| 11 | 32 | Male | Negative | Negative | Negative | Negative |
| 12 | 20 | Male | Negative | Negative | Negative | Negative |
| 13 | 18 | Female | Negative | Negative | Negative | Negative |
| 14 | 31 | Female | Negative | Negative | Negative | Negative |
| 15 | 47 | Male | Negative | Negative | Positive | Positive |
| 16 | 29 | Male | Negative | Negative | Negative | Positive |
| 17 | 14 | Male | Negative | Negative | Negative | Negative |
| 18 | 63 | Male | Negative | Negative | Positive | Positive |
| 19 | 67 | Male | Negative | Negative | Negative | Negative |
| 20 | 35 | Female | Negative | Negative | Negative | Negative |
| 21 | 27 | Male | Negative | Negative | Negative | Negative |
| 22 | 48 | Male | Negative | Negative | Negative | Negative |
| 23 | 17 | Male | Negative | Negative | Negative | Positive |
| 24 | 30 | Male | Negative | Negative | Positive | Positive |
| 25 | 59 | Male | Negative | Negative | Negative | Positive |
| 26 | 31 | Male | Negative | Negative | Negative | Negative |
| 27 | 42 | Male | Negative | Negative | Negative | Negative |
| 28 | 27 | Male | Negative | Negative | Negative | Negative |
| 29 | 17 | Male | Negative | Negative | Negative | Negative |
| 30 | 33 | Male | Negative | Negative | Negative | Negative |
| 31 | 47 | Male | Negative | Negative | Negative | Negative |
| 32 | 32 | Male | Negative | Negative | Negative | Negative |
| 33 | 57 | Male | Negative | Negative | Negative | Negative |
| 34 | 49 | Female | Negative | Negative | Negative | Negative |
| 35 | 48 | Male | Negative | Negative | Negative | Negative |
| 36 | 53 | Male | Negative | Negative | Positive | Negative |
| 37 | 27 | Male | Negative | Negative | Negative | Negative |
| Contd. | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 38 | 77 | Female | Negative | Negative | Negative | Negative |
| 39 | 35 | Male | Negative | Negative | Negative | Positive |
| 40 | 21 | Male | Negative | Negative | Negative | Negative |
| 41 | 26 | Female | Negative | Negative | Negative | Negative |
| 42 | 20 | Male | Negative | Negative | Positive | Negative |
| 43 | 29 | Male | Negative | Negative | Negative | Negative |
| 44 | 50 | Female | Negative | Negative | Negative | Positive |
| 45 | 11 | Male | Negative | Negative | Negative | Negative |
| 46 | 25 | Male | Negative | Negative | Negative | Negative |
| 47 | 35 | Male | Negative | Negative | Negative | Negative |
| 48 | 38 | Male | Negative | Negative | Negative | Negative |
| 49 | 14 | Male | Negative | Negative | Negative | Negative |
| 50 | 24 | Male | Negative | Negative | Positive | Positive |
| 51 | 34 | Male | Negative | Negative | Negative | Negative |
| 52 | 40 | Female | Negative | Negative | Negative | Negative |
| 53 | 49 | Male | Negative | Negative | Positive | Positive |
| 54 | 53 | Male | Negative | Negative | Negative | Negative |
| 55 | 64 | Male | Negative | Negative | Negative | Positive |
| 56 | 26 | Male | Negative | Negative | Negative | Negative |
| 57 | 26 | Male | Negative | Negative | Negative | Negative |
| 58 | 24 | Male | Negative | Negative | Negative | Positive |
| 59 | 21 | Male | Negative | Negative | Negative | Negative |
| 60 | 14 | Male | Negative | Negative | Negative | Negative |
| 61 | 43 | Female | Negative | Negative | Negative | Negative |
| 62 | 36 | Male | Negative | Negative | Negative | Negative |
| 63 | 31 | Male | Negative | Negative | Negative | Negative |
| 64 | 25 | Male | Negative | Negative | Positive | Negative |
| 65 | 28 | Male | Negative | Negative | Negative | Positive |
| 66 | 15 | Male | Negative | Negative | Negative | Negative |
| 67 | 16 | Male | Negative | Negative | Negative | Negative |
| 68 | 23 | Male | Negative | Negative | Negative | Negative |
| 69 | 79 | Male | Negative | Negative | Positive | Positive |
| 70 | 34 | Female | Negative | Negative | Negative | Negative |
| 71 | 66 | Male | Negative | Negative | Negative | Negative |
| 72 | 47 | Male | Negative | Negative | Negative | Negative |
| 73 | 72 | Male | Negative | Negative | Negative | Positive |
| 74 | 29 | Male | Negative | Negative | Negative | Negative |
| 75 | 19 | Male | Negative | Negative | Negative | Negative |
| 76 | 59 | Male | Negative | Negative | Negative | Negative |
| 77 | 47 | Male | Negative | Negative | Positive | Positive |
| 78 | 35 | Male | Negative | Negative | Negative | Positive |
| Contd. | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 79 | 44 | Female | Negative | Negative | Negative | Negative |
| 80 | 20 | Male | Negative | Negative | Negative | Negative |
| 81 | 30 | Male | Negative | Negative | Negative | Negative |
| 82 | 37 | Male | Negative | Negative | Negative | Negative |
| 83 | 43 | Male | Negative | Negative | Negative | Negative |
| 84 | 26 | Male | Negative | Negative | Negative | Positive |
| 85 | 44 | Male | Negative | Negative | Negative | Negative |
| 86 | 74 | Male | Negative | Negative | Negative | Negative |
| 87 | 49 | Male | Negative | Negative | Negative | Negative |
| 88 | 14 | Male | Negative | Negative | Negative | Negative |
| 89 | 44 | Male | Negative | Negative | Negative | Positive |
| 90 | 29 | Female | Negative | Negative | Negative | Negative |
| 91 | 73 | Male | Negative | Negative | Negative | Negative |
| 92 | 42 | Male | Negative | Negative | Positive | Positive |
| 93 | 51 | Male | Negative | Negative | Positive | Negative |
| 94 | 41 | Male | Negative | Negative | Negative | Negative |
| 95 | 16 | Male | Negative | Negative | Negative | Negative |
| 96 | 33 | Male | Negative | Negative | Negative | Negative |
| 97 | 56 | Female | Negative | Negative | Negative | Negative |
| 98 | 39 | Female | Negative | Negative | Negative | Negative |
| 99 | 23 | Female | Negative | Negative | Negative | Positive |
| 100 | 19 | Male | Negative | Negative | Negative | Negative |

## Appendix V

## Statistical analysis

Calculation No.1

Out of 150 Auramine positive cases 123 were male and 27 were female

| | |
|---|---|
| Total patient | 150 |
| Male | 123 |
| Female | 27 |

Null hypothesis (HO) $\mu 1 = \mu^2$

Alternate hypothesis (HA) $\mu 1 =/= \mu^2$

| O | E | O-E | $(O-E)^2$ | $(O-E)^2/E$ |
|---|---|---|---|---|
| 123 | 75 | 48 | 2304 | 30.72 |
| 27 | 75 | -48 | 2304 | 30.72 |
| | | | | 61.44 |

$X^2$ obs = 61.44

$X^2$ tab = (0.05, 1 d f) 3.84

As $x^2$ obs is greater than $x^2$ tab, hence the alternate hypothesis is accepted. Auramine positive evidence of tuberculosis is greater in male than female is statistically significant.

Calculation No.2

Out of 136 culture positive cases 111 were male and 25 were female

| | |
|---|---|
| Total patient | 136 |
| Male | 111 |
| Female | 25 |

Null hypothesis (HO) $\mu 1 = \mu^2$

Alternate hypothesis (HA) $\mu 1 =/= \mu^2$

| O | E | O-E | $(O-E)^2$ | $(O-E)^2/E$ |
|---|---|---|---|---|
| 111 | 68 | 43 | 1849 | 27.19 |
| 25 | 68 | -43 | 1849 | 27.19 |
| | | | | 54.38 |

$X^2$obs = 54.38

$X^2$ tab = (0.05, 1 d f) 3.84

As $x^2$ obs is greater than $x^2$ tab, hence the alternate hypothesis is accepted. Culture positive evidence of tuberculosis is greater in male than female is statistically significant.

Calculation No.3

Out of 150 X-Ray positive cases 123 were male and 27 were female

| | |
|---|---|
| Total patient | 150 |
| Male | 123 |
| Female | 27 |

Null hypothesis (HO) $\mu 1 = \mu^2$

Alternate hypothesis (HA) $\mu 1 =/= \mu^2$

| O | E | O-E | $(O-E)^2$ | $(O-E)^2/E$ |
|---|---|---|---|---|
| 123 | 75 | 48 | 2304 | 30.72 |
| 27 | 75 | -48 | 2304 | 30.72 |
| | | | | 61.44 |

$X^2$ obs = 61.44

$X^2$ tab = (0.05, 1 d f) 3.84

As $x^2$ obs is greater than $x^2$ tab, hence the alternate hypothesis is accepted. X-Ray positive evidence of tuberculosis is greater in male than female is statistically significant.

Calculation No.4

Out of 134 Z-N stain positive cases 111 were male and 25 were female

| | |
|---|---|
| Total patient | 134 |
| Male | 110 |
| Female | 24 |

Null hypothesis (HO) $\mu 1 = \mu^2$

Alternate hypothesis (HA) $\mu 1 =/= \mu^2$

| O | E | O-E | $(O-E)^2$ | $(O-E)^2/E$ |
|---|---|---|---|---|
| 110 | 67 | 43 | 1849 | 27.59 |
| 24 | 67 | -43 | 1849 | 27.59 |
| | | | | 55.18 |

$X^2$ obs = 55.18

$X^2$ tab = (0.05, 1 d f) 3.84

As $x^2$ obs is greater than $x^2$ tab, hence the alternate hypothesis is accepted. Z-N stain positive evidence of tuberculosis is greater in male than female is statistically significant.

Photograph 1 Z-N stained sputum smear showing red AFB of MTB (100X)

Photograph 2 Auramine stained sputum smear showing glowing MTB (40X)

Photograph 3 Microscopic observation of Z-N stained slide

Photograph 4 Observation of Auramine stained slide by fluorescent microscope

Photograph 5 Culture of *Mycobacterium* on LJ medium