CHAPTER I

1. INTRODUCTION

Tuberculosis (TB) constitutes a major public health problem in most developing countries of the world. It accounts for the largest burden of mortality due to any infectious agent worldwide. The disease is preventable and treatable but early diagnosis, effective treatment and successful cessation of transmissions are required for effective control of tuberculosis. There are many diagnostic methods recommended by World Health Organization (WHO) and all of them are not available or not feasible to use in developing countries (WHO, 2007; NTC, 2006).

The simplest diagnostic technique for tuberculosis is microscopy. The active tuberculosis can be diagnosed mostly by sputum staining. However, other specimens like bronchial washing, body fluid, pus, urine, CSF etc can also be stained and observed for acid fast bacilli.

In nearly all clinical circumstances in high prevalence areas, finding acid-fast bacilli in stained sputum is highly specific and therefore, is the equivalent of a confirmed diagnosis (Hopewell *et al.*, 2006).

Detection of smear positive cases identifies those cases that are infectious and contributing substantially to the transmission of disease (Harries *et al.*, 1998). Acid-fast bacilli (AFB) microscopy which is a means of detecting and screening of TB has been used worldwide and it remains as a mainstay of case finding (Barez *et al.*, 1995). The finding of AFB in sputum establishes a presumptive diagnosis of TB and is crucial to guide treatment, to limit person to person spread and to assess the degree of the activity of the disease (Behr *et al.*, 1999).

Acid -fast staining technique is the fastest, easiest, and least expensive tool for the rapid identification of potentially infectious TB patients (Murray *et al.*, 2003). The importance of the method is further exemplified by the fact that in low-income countries, (like Nepal) the

definitive diagnosis of TB still relies solely on the presence of AFB in stained smears. Though the specificity of acid-fast microscopy is excellent for mycobacterial species, the sensitivity is not optimal.

The sensitivity of microscopy is influenced by numerous factors, such as the prevalence and severity of disease, the type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, the staining technique, and the quality of the examination (Deun, 1998). However, the specificity of smear examination methods should be interpreted with caution because it does not allow differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT).

Presently, two types of acid-fast stains are commonly used in clinical Mycobacteriology laboratories. One type is carbol fuchsin (Ziehl-Neelsen [ZN] or Kinyoun methods), and the other is fluorochrome either auramine phenol (AP) or auramine-rhodamine (AR). The present ZN method has evolved from Koch's original alkaline methylene blue-based method following significant modifications. ZN stain is commonly used throughout the world and still remains the standard method against which new tests must be measured. Methods applying fluorochrome have been used for acid-fast staining since the introduction of an auramine O and an AR based methods. Both techniques are based on the relatively unique property of *Mycobacterium* to retain the primary stain even after exposure to strong mineral acid or acid-alcohol, hence the term, acid-fast bacilli (Sonnenwirth and Jarett, 1990).

In developing countries like Nepal, the standard method culture and sensitivity testing is only available at central level. The only method that all TB laboratories are able to conduct at provincial and district level is sputum smear microscopy. Although AFB smears are less sensitive than cultures, the procedure for collecting and examining samples is relatively simple,

rapid and inexpensive (Aydan, 2007). Expansion and enhancement of DOTS have been implemented in Nepal by Health Ministry's National Integrated Programme to combat leprosy and TB since 1996. In Nepal, the percentage of incident smear positive cases being notified was only 45% in 2008 (WHO, 2009). Concerted effort should be made to ensure the effective microscopy.

In addition to focusing on developing new technologies to support case finding, existing technologies, approaches and recommendations need to be examined carefully to determine whether they are being utilized optimally and to identify areas in which improvements in efficacy and efficiency can be made (Mase, 2007). Communities and health centers have minor roles in carrying out TB services because health workers have insufficient experience with diagnostic testing. Therefore, under the present circumstances sputum microscopy is nevertheless a rapid way of detecting the most contagious patients, and its specificity is high. If the sensitivity could be increased it would be even more useful diagnostic tool in the developing world (Angeby *et al.*, 2000).

In sputum smear microscopy, ZN is the most commonly used technique, because of its simplicity and low cost. In fluorescence method, the specific staining solution contains a fluorochrome (auramine o), which makes the TB bacilli 'fluoresce' when viewed under fluorescence microscopy. Fluorochrome stained smears are generally scanned at 250x to 450x while fuchsin stained smears of ZN method are examined at 1000x (Mendoza, 1987). The aim of our study was to compare the most popularly used ZN stain with Fluorescence (auramine phenol) staining techniques in different clinical specimens.

CHAPTER II

2. OBJECTIVES

2.1 General Objectives

To compare Ziehl-Neelsen Microscopy with Fluorescence Microscopy for the detection of acid fast bacilli in different clinical specimens.

2.2 Specific Objectives

i. To compare ZN Microscopy with Fluorescence Microscopy for detection of acid–fast bacilli in sputum sample.
ii. To compare ZN Microscopy with Fluorescence Microscopy for detection of acid–fast bacilli in extra pulmonary tuberculosis samples.
iii. To determine most appropriate method of staining in a laboratory set up.

CHAPTER III

3. LITERATURE REVIEW

3.1 Epidemiology of Tuberculosis

Tuberculosis (TB) constitutes a major public health problem in most developing countries of the world. It accounts for the largest burden of mortality due to any infectious agent worldwide (WHO, 2007). TB infection is caused primarily by *Mycobacterium tuberculosis* and less commonly by *M. africanum* or *M. bovis* (Collee *et al.*, 1996). This disease has the potential to infect virtually every organ, most importantly the lungs due to dissemination via lympho-hematogenous route (Haas, 2000).

TB is an ancient disease which continues to haunt us even we step into the next millennium. It has also been estimated that someone in the world is infected with TB every second, nearly one percent of the world population is infected with TB every year and according to the World Health Organization (WHO), one-third of the world's population is infected with organisms of the *M. tuberculosis* complex, with about 10 million cases of active TB disease reported each year, leading to 3 million deaths annually (Mohan and Sharma, 2001; Dollin *et al.*, 1994). In developing countries 95% of TB cases and 98% of TB deaths occurs and these deaths comprise 26% of all avoidable deaths. In developing countries 75% of TB cases are in the economically productive age group (15-50) (Sharma, 2001).

Globally, there were an estimated 9.27 million incident cases of TB in 2007. This is an increase from 9.24 million cases in 2006, 8.3 million cases in 2000 and 6.6 million cases in 1990. Most of the estimated numbers of cases in 2007 were in Asia (55%) and Africa (31%), with small proportions of cases in the Eastern Mediterranean Region (6%), the European Region (5%) and the Region of the Americas (3%). The five countries that rank first to fifth in terms of total numbers of cases in 2007 are India (2.0 million), China (1.3 million), Indonesia (0.53 million), Nigeria (0.46 million) and South Africa (0.46

million). Of the 9.27 million incident TB cases in 2007, an estimated 1.37 million (15%) were HIV-positive; 79% of these HIV-positive cases were in the African Region and 11% were in the South-East Asia Region (WHO, 2009).

TB is one of the major public health problems in Nepal. According to WHO, Nepal is 27th highest TB burden country in the world, with estimated annual risk of infection of 1.8% (WHO, 2007). About 45% of the total population is infected with TB, out of which 60% are adult. In Nepal, 80,000 people have TB. Every year 40,000 people develop active TB, of whom 20,000 have infectious (pulmonary disease) sputum positive TB, and 5,000 to 7,000 people die from TB every year (DoHS, 2009). The prevalence of tuberculosis per 100,000 population in 2007 was 240 and incidence of TB per 100,000 population per year was 173 (WHO, 2009).

3.1.1 Transmission

TB is a serious disease, and a highly contagious one. Transmission is most likely when there is prolonged close contact between a susceptible person and a person who has an active case of TB, but that contact does not have to be intimate (Salyers, 2002). Tuberculosis is transmitted mainly by droplet infection and droplet nuclei generated by sputum positive patients with pulmonary tuberculosis by coughing, sneezing and talking. To transmit infection, the particles must be fresh enough to carry a viable organism. The airborne particles (droplet nuclei) 1-5µm in size are kept “suspended” by normal air current infection occurs when a susceptible person inhale the droplet nuclei. One air borne particle contains 1-10 bacilli. Patients who excrete 10,000 or more tubercle bacilli per ml of sputum are the main source of infection to other (Groothius and Yates, 1991). Coughing generates the largest number of droplet nuclei. Transmission generally occurs indoors, where droplet nuclei can stay in the air for a long time (WHO, 2008). Tuberculosis is not transmitted by fomites, such as dishes and other articles used by the patients. Sterilization of these articles is, therefore, of little or no value. Patients

with extra pulmonary tuberculosis or smear negative tuberculosis constitute a minimal hazard for transmission of infection (WHO, 2008).

3.1.2 Risk of Infection

Risk of infection is depends on the extent of exposure to droplet nuclei and susceptibility to infection. Risk of infection of a susceptible individual is especially increases in crowded and poor ventilated area (Basnet, 1998). The risk of transmission of infection from a person with sputum smear negative PTB is low and with extra pulmonary TB is even lower (NTC, 2007).

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

Risk of infection are dependent on the several predisposing factors, such as: old age, alcoholism, diabetes, neoplastic disease, malnutrition, immunosuppressive drugs, stress and drug induced, congenital or acquired immunodeficiency including HIV infection (Groothius and Yates, 1991). As immunity wanes through aging or immune suppression, the dormant bacteria become reactive causing an outbreak of disease often many decades after the initial infection. Immunosuppression due to disease of drug therapy is a major predisposing factor for the development of mycobacterial disease (Groothius and Yates, 1991). HIV infection appears to increase the risk greatly and shorten the interval for the development of TB disease (Elliot *et al.*, 1999).

3.2 Tuberculosis and HIV Co-Infection

HIV pandemic has a major impact on tuberculosis. Infection with HIV greatly increases the risk of developing tuberculosis and accelerates its progress (Prasanthi *et al.*, 2005). It directly

attacks the critical immune mechanisms involved in protection against tuberculosis. In the early stages of HIV infection, when CMI is only partially compromised, pulmonary tuberculosis presents typically as upper lobe infiltrates and cavitation with high bacillary load in the sputum, whereas in the late stages, primary tuberculosis like pattern with diffuse interstitial and military infiltrates, little or no cavitation is seen resulting in paucibacillary picture of sputum (Raviglione *et al.,* 2002; Kaufmann, 2001). Progression to AIDS is also accelerated in those co infected and HIV has significant effect on the risk of relapse of tuberculosis (Cheesbrough, 2006).

Among the 9.27 million incident cases of TB in 2007, an estimated 1.37 million incident cases of HIV positive (14.8%) and 456,000 deaths from TB among HIV-positive people. The global number of incident HIV-positive TB cases is estimated to have peaked in 2005, at 1.39 million. In 2007, as in previous years, the African Region accounted for most (79%) HIV-positive TB cases, followed by the South-East Asia Region (mainly India) with 11% of total cases South Africa accounted for 31% of cases in the African Region (WHO, 2009). The prevalence of HIV is also rising rapidly in Nepal, 2.4% of tuberculosis patients also had HIV infection in 2006 (DoHS, 2009).

3.3 Control and Prevention of Tuberculosis

Control measures target the interruption of the disease transmission. For efficient control, the epidemiology of the disease has to be understood. As disease being transmitted through airborne route, the early treatment of disease personnel and the human link in the transmission can be controlled by taking personal precautions.

3.3.1 Prevention of Human Infection

TB disease may remain contagious until the person has been on appropriate treatment for several weeks. Thus the most important way to stop the spread of the tuberculosis is to cover the mouth and nose when coughing and to take prescribed medicine as directed. Persons with disease should have

respiratory precautions until symptoms are improved and there is documentation of adequate response to therapy by three consecutive negative sputum smears collected on different days. All household and close contracts of a person with active TB disease should be screened, using the Mantoux skin test for evidence of infection. All contacts with evidence of infection should be evaluated for treatment by a physician. All high risk populations should be TB skin tested routinely. It is important to note that a person with TB infection, but not disease, cannot spread the infection others, since there are no bacteria in the sputum (Ohio, 2009).

Early case detection and treatment are the most important control measures. BCG vaccination is widely used to prevent the tuberculosis and is safe enough to give to infants. Unfortunately, the effectiveness of BCG in preventing tuberculosis is controversial. The vaccine has some efficacy in preventing tuberculosis in children but does not prevent reactivation of preexisting infection in older people (Salyers, 2002).

3.3.2 Control of the Reservoir

Human being only known reservoir of tuberculosis, early case detection and prompt treatment is only the way to control disease.

3.3.2.1 Treatment

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

Five drugs regard as essential in the treatment of TB: Isoniazid (INH), Rifampicin (RFP), Pyrazinamide (Z/PZA), Streptomycin (SM) and Ethambutol (EMB). Thioacetazone (T) is also used to supplement INH in many developing countries because of its low cost (WHO, 2007). Three main

properties of anti-TB drugs are: bactericidal ability, sterilizing ability and the ability to prevent resistance. The anti-TB drugs posses these properties to different extents. INH and RFP, the most powerful bactericidal drugs, are active against all populations of TB bacilli. PZA and SM are also bactericidal against certain populations of TB bacilli. PZA is active in an acid environment against TB bacilli inside macrophages. SM is active against rapidly multiplying extra cellular TB bacilli. EMB and T are bacteriostatic drugs, used in association with more powerful bactericidal drugs to prevent the emergence of resistant (WHO, 2007).

DOTS (Directly observed treatment short course), a TB control strategy, pioneered by international union against TB and lung disease (IUATLD) and recommended by WHO to ensure cure by providing the most effective medicine and confirming that it documented to be effective worldwide on a programme basis. This strategy controls TB by giving drugs to patients under direct observation of health workers. This means that health workers support the patients during the course of treatment and watch the patient swallow the tablets. DOTS has been found 100% effective to cure TB and to prevent MDR. Only DOTS ensure cure of diagnosed TB patients. It can also prevent relapse and death. DOTS introduced in Nepal in 1996, and successfully implemented throughout the country since April 2001. By July 2008 DOTS had been expanded to 1,079 treatment centers with 3,147 sub-centers. The treatment success rate in DOTS is now 88% in Nepal (DoHS, 2009).

3.4 Causative Agent of Tuberculosis

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

between *M. bovis and M. tuberculosis* and also cause human tuberculosis and mainly found in equatorial Africa (Chakraborty, 2001).

MOTT exists as saprophyte of soil and water and occasionally causes opportunistic disease in human. Infection caused by this organism is known as mycobacterioses. Four main types of diseases are caused by MOTT. They are skin lesions followed by traumatic inoculation of bacteria, localized lymphadenitis, and tuberculosis like pulmonary lesions and disseminated diseases (Chakraborty, 2001).

3.4.1 Morphology of *M. tuberculosis*

M. tuberculosis is straight or slightly curved rod usually 1-4µm×0.3-0.6µm in size. It may be arranged singly or small clumps. It is non-motile, non-sporing and non-capsulated, acid-fast and Gram positive bacterium. Cells of *M. tuberculosis* are often arranged as 'serpentine cords' & it demonstrates beaded or barred staining (Grange, 1998). Non-acid fast rods and granules from young culture are also reported and when they are injected into susceptible animals, they produce tuberculosis. Perhaps these granules are non-acid fast form of tubercle bacilli. These bacilli are called Much's granules (Chandrasekhar, 2007).

3.5 Cellular Structure and Acid Fast Property of Mycobacterium

Unlike cell walls of other gram positive bacteria, the cell wall of mycobacterium species consists not only of peptidoglycan but also of a number of unusual glycolipids that make mycobacteria impervious to mild staining procedures such as Gram stain. In addition to peptidoglycan, the mycobacterial cell wall contains glycolipids such as the arabinogalactan lipids and mycolic acids; a type of lipid found only in cell wall of mycobacterium and *Corynebacterium* species (Salyers, 2002).

A prominent feature of the outer surface of mycobacteria is its high content of lipid, which accounts for up to 60% of the cell wall weight and which contributes to several biological

features including the hydrophobicity of mycobacteria, the tendency of mycobacteria to form clumps or cords, the resistance of mycobacteria to common lysis procedure and the ability of mycobacteria to survive intracellurlarly. Lipoarabinomannan (LAM) is structurally and functionally related to the O – antigenic lipopolysaccharides of other bacteria. Biological activities of LAM include strong seroreactivity, inhibition of interferon gamma mediated activation of macrophages, scavenging reactive oxygen intermediate and suppression of T cell proliferation (Grange, 1998).

The mycobacterial mycolic acid is -branched, - hydroxyl fatty acids with 60-90 carbon atoms in the primary chain. Each species of mycobacterium appears to synthesize a unique set of mycolic acid and has been exploited for speciating mycobacteria (Grange, 1998). The most distinctive property of mycobacterial cell is its unusual cell wall, a multilayered structure that contains N- glycolylmuramic acid instead of N-acetylmuramic acid and very high lipid content. Because of this distinctive property within the genus mycobacterium, it has its characteristic staining (Salyers, 2002).

Mycobacteria are difficult to stain. The large amounts of lipids present in their cell walls makes them impermeable to the gram stain, and appearance of mycobacteria in a gram stained specimen may be variable. Mycobacteria are able to form stable complexes with certain aryl methane dyes such as fuchsin and auramine O. Although the exact nature of the acid-fast staining reaction is not completely understood, phenol in the primary stain allows penetration of the stain. The cell wall mycolic acid residues retain the primary stain even after exposure to acid-alcohol or strong mineral acids. A counter stain is employed to highlight the stained organisms for easier microscopic recognition. The acid fast nature of an organism can be determined by several methods. In carbol fuchsin staining procedures AFB appear red against a blue or green background (Collee *et al.*, 1996). Fluorescent staining is regarded as a more reliable method due to more

intensive binding of mycolic acids of the bacilli to phenol auramine, and so the bacilli stand out sharply against black background to allow rapid and accurate screening under low power objective (Ponticeellio *et al.*, 2001).

3.6 Pathogenesis of Tuberculosis

The tubercle bacillus owes its virulence to its ability to survive within the macrophage rather than to the production of a toxic substance. The immune response to the bacillus is of the cell-mediated type which, depending on the type of T- helper cells involved, many either lead to protective immunity and resolution of the disease or to tissue destroying hypersensitivity reactions and progression of the disease process (Grange, 2002).

3.6.1 Entry of Bacteria

Tuberculosis is serious disease and a highly contagious one. The disease is spread from person to person by aerosols. The tiny droplets generated during coughing, talking, splitting are small enough that it bypass the defense of respiratory tract and thus organism rich directly into the lungs (Salyers, 2002).

3.6.2 Incubation Period

Evidence of infection (a positive skin test) usually occurs 4-12 weeks after exposure. The most common period for developing clinical disease is 12-24 months after infection (Ohio, 2009). However, the development of disease depends upon the closeness of contact, extent of the disease and sputum positivity of the source case (dose of infection) and host-parasite relationship. Thus the incubation period may be weeks, months or years (Park, 2000).

3.6.3 Immunology

An important defense of the lung is the alveolar macrophage. *M. tuberculosis* has evolved the ability to survive and multiply in inactivated macrophages. Activated macrophages can kill the bacteria, however. Thus, the ability of a person to mount a rapid and effective activated macrophage response determine whether the outcome of exposure will be symptomatic disease or not. The initial interaction between

M. tuberculosis and macrophages elicits both a T-helper (CD4+) and a cytotoxic T-cell (CD8+) response. The CD4+ T cells stimulate antibody production, but antibodies against tuberculosis are useless since the bacteria are serum resistant and can multiply inside phagocytes. The main contribution of the CD4+ T cells to controlling an incipient infection seems to be production of IFN gamma, which stimulates macrophage activation. (CD8+ T cells also release IFN gamma but a lesser extent.) IFN gamma also stimulates endothelial cells to binds T cells, thus triggering their movement out of the blood vessel and into adjacent tissues so that they can converse on the infected area. CD8+ T cells kill infected phagocytes that have not succeeded in destroying the bacteria so that the bacteria can be ingested and killed by activated macrophages. In a healthy adults exposed to relatively low number of bacteria, activated macrophages generally appear early enough to stop the infection before appreciable damage is done to the lung. Such people become skin test positive but do not develop symptomatic TB. In an infant or in an adult who cannot mount a rapid and effective T-cell response, activated macrophages do not appear until much later in the disease process, and the bacteria continue to multiply in lung macrophages. Since the phagocytic cells are not clearing the infection, new T cells, PMNs, and macrophages continue to be attracted to the area and accumulate around the sites where bacteria are growing. Macrophages in the vicinity of the bacteria fuse to form giant cells and a layer of macrophages and T cells forms around a growing focus of damaged tissue containing the bacteria (Salyers, 2002).

In some cases, although the phagocytes are not able to kill the bacteria, the T cells and macrophages are successful in walling off the growing lesion with a thick fibrin coat. The walled off lesion is called a tubercle. Tubercles eventually calcify, giving rise to the hard edged lesions visible in chest radiograms. The cell mediated (T cells plus phagocytes) response that produces tubercles is called agranulomatous response because the tubercles appear

macroscopically as granulomas (Salyers, 2002).

Phagocytes trying unsuccessfully to kill the bacteria cause considerable damage to lung tissue, both by releasing lysozomal enzymes and by producing TNF . TNF causes tissue damage and is probably also responsible for the weight loss that occur in people with TB. An important characteristic of lesion that develops during a case of TB is the consistency of their contents. Initially, the areas where bacteria are dividing have a thick, cheese like consistency (caseous necrosis). As bacteria continue to divide and phagocytes continue to enter the area, the necrotic region becomes much more liquid. Since a liquid is much more easily rendered into aerosols than the thicker caseous material, a person with liquefied lesions is much more contagious than a person whose lesions are in the caseous necrosis stage. Also, the thick consistency of a caseous lesion prevents bacterial movement out of the area. Bacteria in a liquefied lesion can escape from the lesion more readily and spread to other parts of the body, causing the disseminated form of the disease (miliary TB) (Salyers, 2002).

A person who has mounted an immune response that stops bacterial growth and prevents the development of severe symptomatic disease is not necessarily out of danger. Walled off lesions may contain live bacteria. An unusual feature of *M. tuberculosis* is its ability to survive for decades in such lesions. Later in life, suppression of the immune system (example; by cancer, immunosuppressive drugs, AIDS) may allow the bacteria to break out of the lesion and begin to multiply again. This form of the disease, called reactivation TB, is identical to primary TB in its infectiousness and in the damage it can cause (Salyers, 2002).

Survival in Phagocytes

A key virulence property of *M. tuberculosis* is its ability to multiply inside monocytes and macrophages. It can also multiply inside monocytes and macrophages. It can also invade PMNs, but macrophages, especially the activated ones, are the key to

development of the infection. Complement mediated opsonization is not a way for bacteria to enter the phagocytic cells because levels of complement in the lungs are only 1-3% of those in blood. Instead *M. tuberculosis* binds directly to macrophage surface protein CR3, the normal receptor for iC3b. The bacteria also bind to other macrophage receptor proteins, such as CR4. Binding is followed by internalization of the bacteria in a vesicle. Macrophages that take up *M. tuberculosis* have a reduced ability to carry out phagolysozome fusion, because the bacteria prevent the interior of the vesicle from acidifying. Moreover, infected macrophages have a reduced oxidative brust. Another effect of the bacteria on the macrophages is to reduce their production of IL – 12, a cytokine that stimulates the Th1 response (Salyers, 2002).

3.7 Clinical Forms of Tuberculosis

3.7.1 Pulmonary Tuberculosis

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

Primary Infection

Primary infection occurs in people who have not had any previous exposure to tubercle bacilli. Droplet nuclei, which are inhaled into the lungs, are so small that they avoid the mucocilliary defences of the bronchi and lodge in the terminal alveoli of the lungs. Infection begins with multiplication of tubercle bacilli in the lungs. The resulting lesion is the Ghon focus. Lymphatics drain the bacilli to the hilar lymph nodes. The Ghon focus and the related hilar lymphadenopathy form the primary complex. Bacilli may spread in the blood from the primary complex throughout the body. The immune response (delayed hypersensitivity and cellular immunity) develops about 4-6 weeks after the primary infection. The size of the infecting dose of bacilli and the strength of the immune response determine what happens next. In most cases, the immune response stops the

multiplication of bacilli. However, the few dormant bacilli may persist. A positive tuberculin skin test would be the only evidence of infection. In a few cases the immune response is not strong enough to prevent multiplication of bacilli, and disease occurs within a few months (WHO, 2007).

Post-Primary Tuberculosis

Post-primary TB occurs after a latent period of months or years following primary infection. It may occur either by reactivation of the dormant tubercle bacilli acquired from a primary infection or by re-infection. Reactivation means that dormant bacilli persisting in tissues for months or years after primary infection starts to multiply. This may be in response to trigger, such as weakening of the immune system by HIV infection. Re-infection means a repeat infection in a person who has previously had a primary infection.

The immune response of the patient results in the pathological lesion that is characteristically localized, often with extensive tissue destruction and cavitations. Post-primary TB usually affects the lungs but can involve any part of the body. The characteristics features of the post-primary PTB are the following: extensive lung destruction with cavitation; positive sputum smear; upper lobe involvement, usually no intrathoracic lymphadenopathy. Patients with these lesions are the main transmitters of infection in the community (WHO, 2007).

3.7.2 Extra Pulmonary Tuberculosis

TB can affect any organ and tissue of the body. Most, if not all, extra pulmonary lesions result by haematogenous spread of the organism from a primary focus which is not always detected (Chakraborty, 2001). Common forms of extra pulmonary TB include the following: lymphadenopathy, pleural effusion TB, meningitis, miliary, intestinal, bone, urogenital, skin and eye TB (RNTCP, 2005). Patients usually present with constitutional features (fever, night sweats, weight loss) and local features related to the site of diseases. The

highest rate of infection of extra pulmonary tuberculosis cases in immunocompromised states associated with old age, renal failure(including dialysis and transplant patients),cirrhosis, malnutrition, hematologic malignancies and HIV/AIDS. The study on Thailand showed that 12.5% of EPTB patients were co infected with HIV (Wiwatworapan, 2008). Other research also showed similar results (Solomon *et al.*, 2006; Kwara *et al.*, 2005)

3.8 Diagnostic Method for Tuberculosis

3.8.1 Clinical Diagnosis

Clinically, pulmonary tuberculosis is chiefly present with persistent cough for 3 or more weeks, haemoptysis, shortness of breath and chest pain, loss of appetite and loss of weight, malaise, fatigue, night sweat and fever (WHO, 2008). More specific symptoms will depend on the organs involved and the extent of disease process (Ohio, 2009). The disease in the children is not easy to diagnose since there is hardly any productive cough. The symptoms are usually weight loss and enlargement of the lymph glands which may cause obstruction of the bronchi and emphysema. Clinical signs and symptoms develop in only a small proportion (5-10%) of infected healthy people.

3.8.2 Radiological Diagnosis

Chest X ray can also help in the detection of the pulmonary tuberculosis but they don't allow etiological diagnosis. X-ray suggesting tuberculosis include upper lobe infiltrate, cavity infiltrate and hilar or paratracheal adenopathy but this finding has low positive predictive value and specificity. In a Danish study, X-ray changes thought to be due to TB showed positive predictive values and sensitivity for TB were 61% and 67% respectively. Also in a South African study of the diagnostic accuracy of X-ray in children, the results yield a sensitivity of 38.8% and a specificity of 74.4% compared to culture for the diagnosis of pulmonary TB using standard radiographs (NCC, 2005). In

many patients with primary progressive tuberculosis and those with HIV infection radiographic findings are subtler and can include lower lobe infiltrates or a miliary pattern. Miliary lesions, which are small granulomas, resemble millet seeds spread throughout the lung fields (Bloom, 1994).

3.8.3 Laboratory Diagnosis

Active tuberculosis (TB) is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other body sites (extra pulmonary TB). Although many new (molecular) diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture on Lowenstein-Jensen medium are still the "gold standards" for the diagnosis of active TB and, especially in low-resource countries, the only methods available for confirming TB in patients with a clinical presumption of active disease. AFB smear microscopy is rapid and inexpensive and thus is a very useful method to identify highly contagious patients. Culture is used to detect cases with low mycobacterial loads and is also requested in cases at risk of drug-resistant TB for drug susceptibility testing, or in cases where disease due to another member of the *Mycobacterium* genus is suspected. AFB smear microscopy and culture can also be used to monitor the effectiveness of treatment and can help to determine when a patient is less likely to be infectious (Waard, 2007).

3.8.3.1 Specimen Handling

The successful isolation of the pathogen requires that the best specimen be properly collected, promptly transported and carefully processed. Many different types of clinical specimens may be obtained for the microbiological diagnosis. If pulmonary TB is suspected, specimens originating from the respiratory tract should be collected, i.e. sputum, induced sputum, broncho-alveolar lavage or a lung biopsy. For the diagnosis of pulmonary TB, three first-morning sputum specimens (not saliva) obtained after a deep, productive cough on non-consecutive days are usually

recommended. Several studies have shown, however, that the value of the third sputum is negligible for the diagnosis of TB, as virtually all cases are identified from the first and/or the second specimen (Crampin *et al.,* 2001; Harries *et al.,* 2000; Ozkutuk *et al.,* 2007; Finch, 1997, Sarin *et al.,* 2001). Before processing, sputum specimens must be classified at the laboratory with regard to their quality, i.e. bloody, purulent, mucopurulent, saliva. In patients who cannot produce it spontaneously, the sputum can be induced by inhalation of hypertonic saline solution. Otherwise, the specimen can be collected from bronchoscopy. Some studies suggest that a single induced sputum specimen is equally effective as bronchoscopy for diagnosing pulmonary TB (Conde *et al.,* 2000). A recent study demonstrated that the most cost-effective strategy is to perform three induced sputum tests without bronchoscopy (MacWilliams, 2002). "Fasting" gastric aspirates is the specimen of choice in the case of young children who cannot cough up phlegm. Gastric lavage fluid must be neutralized with sodium carbonate immediately after collection (100 mg per 5-10 ml specimen) (Waard *et al.*, 2007).

Specimens to be collected for the diagnosis of extra pulmonary disease depend on the site of the disease. The most common specimens received in the laboratory are biopsies, aspirates, pus, urine, and normally sterile body fluids, including cerebrospinal fluid, synovial, pleural, pericardial, and peritoneal liquid. Stool can be collected when intestinal TB is suspected and also in the case of suspected *Mycobacterium avium* infection in AIDS patients. Whole blood and/or bone marrow specimens are collected only if disseminated TB is suspected, mainly in patients with an underlying severe immunosuppressive condition such as AIDS (Grange, 2002; Wiwatworapan, 2008). Bone biopsies are the specimen of choice when skeletal TB is suspected. In general, AFB smear microscopy from body fluids is rarely positive and the whole sediment from concentrated specimens should rather be cultured. In tuberculous pleural effusions, the diagnostic value of the pleural biopsy is

much higher than that of the fluid and, therefore, is the specimen of choice for the diagnosis (Waard *et al.*, 2007). The diagnosis of peritoneal and pericardial TB is difficult and usually requires invasive procedures such as laparoscopy and biopsy. Specimens should be collected in sterile, leak-proof containers and labeled with the patient's name and/or identification number before anti-tuberculosis chemotherapy is started. Induced sputum specimens should be labeled as such because they resemble saliva and may be disregarded at the laboratory. Specimens must be collected aseptically in order to minimize contamination with other bacteria. Blood and other specimens prone to coagulate, including bone marrow, synovial, pleural, pericardial and peritoneal fluids, should be collected in tubes containing sulfated polysaccharides or heparin. Sulfated polysaccharides are the preferred anticoagulants as they enhance the growth of mycobacteria. Heparinized specimens are also satisfactory, but specimens collected in ethylene diamine tetra acetic acid (EDTA) are unacceptable as even trace amounts of this substance inhibit mycobacterial growth. Lymph nodes, skin lesion material, and tissue biopsy specimens should come without preservatives or fixatives and should not be immersed in saline or any fluid. Once in the laboratory, tissue specimens are homogenized in a sterile tissue grinder with a small amount of sterile saline solution before AFB smear staining or culture. Abscess contents or aspirated fluids can be collected in a syringe (Waard, 2007). If renal TB is suspected, the specimen of choice is the first-morning urine, at least 50 ml, obtained by catheterization or from the midstream clean catch on three consecutive days. Urine specimens should be submitted to a decontamination step for mycobacteria prior to cultivation (Waard, 2007).

3.8.3.2 Specimen Transport

Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. This is particularly true for specimens from non-sterile sites, such as sputum. When the transport or the processing is

delayed, specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing. The cetyl pyridinium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick *et al.,* 1975). CPC eliminates the associated flora in sputum specimens and treated specimens should not be submitted to further decontamination prior to cultivation. The detection of AFB with Ziehl- Neelsen staining can be significantly reduced in specimens preserved by this method (Selvakumar, 2004; Selvakumar, 2006). Re-staining seems to increase the detection in sputum specimens transported in CPC solution (Selvakumar, 2005). In addition, CPC inhibits mycobacterial growth, especially when inoculated in culture media including Middlebrook 7H9 and 7H10, which have an insufficient neutralizing activity for this quaternary ammonium compound. Therefore, specimens treated with CPC should be preferentially inoculated in egg-based media (Smithwick *et al.,* 1975). In a comparative study, sodium carbonate was found to be a better preservative of sputum specimens for AFB smear microscopy as well as culture (Bobadilla, 2003). However, no comparative study has been undertaken to confirm this observation.

3.8.3.3 Biosafety

Laboratory diagnosis of TB involves a risk of infection for laboratory personnel. Specimens with high mycobacterial loads, such as sputum or cultures, are often manipulated with limited biosafety measures, especially in low-resource countries. Although only limited information can be found in the literature on the risk of developing TB in laboratory personnel (Kim, 2007), studies in healthcare workers in contact with TB patients clearly show that TB can be considered as an occupational disease (Joshi *et al.,* 2006; Kilinc *et al.,* 2002). Biosafety cabinets are seldom available in developing countries, and safety facilities for working with infectious specimens are limited. The only measure often taught to give any protection against infection with *M. tuberculosis* is processing infectious

specimens behind the flame of a Bunsen burner, that is, the specimens are handled and the smear is prepared with the flame of the Bunsen burner interposed between the operator and the specimen. As far as we know, however, this measure has never been evaluated. Good laboratory practice is required for the protection of laboratory staff from infectious airborne bacilli, i.e. good ventilation, use of laboratory coats, surgical gloves and face masks, hand washing and regular disinfection of the laboratory floor and surfaces, especially benches, with a disinfectant that is active against mycobacteria (Frobes *et al.,* 1998). This disinfectant may be 70 % ethanol or sodium hypochlorite (house bleach) at a concentration of 0.2-0.5 %. Ultraviolet light, emitting rays of wavelength 254 nm, is very effective in killing the tubercle bacillus and other mycobacteria; it is also an additional measure for decontaminating work surfaces and killing airborne microorganisms (Waard, 2007).

3.8.3.4 Microscopy

AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because most mycobacteria grow slowly and culture results become available only after weeks of incubation. In addition, AFB smear microscopy is often the only available diagnostic method in developing countries. Smear staining is based on the high lipid content of the cell wall of mycobacteria which makes them resistant to decolorization by acid-alcohol after the primary staining. To determine that a clinical specimen contains AFB, the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background. The slide is observed under the microscope for the detection of AFB. Several methods can be used for determining the acid-fast nature of an organism.

Two methods, Ziehl-Neelsen and Kinyoun, utilize basic fuchsin in

ethanol for primary staining. In both cases, AFB appears red after decolorization with acid-alcohol. Ziehl-Neelsen is a hot acid-fast stain because the slide has to be heated during incubation with fuchsin. In contrast, Kinyoun staining is a cold acid-fast staining procedure and therefore does not require heating. Kinyoun's cold carbol fuchsin method is inferior to the Ziehl-Neelsen staining (Somoskovi *et al.,* 2001; Deun *et al.,* 2005) and thus Ziehl-Neelsen staining method is used for diagnosis of TB in developing country. In the fluorochrome procedure, primary staining is done with auramine O. The AFB fluoresce yellow against a counter stain of potassium permanganate when observed with a fluorescence microscope. While the reading of fuchsin-stained smears requires 1000x magnification, fluorochrome-stained smears are examined at 250x or 450x. The lower magnification used in this staining method allows the microscopist to observe a much larger area of the smear during the same period of time and thus, fewer fields must be read. This makes the method faster and reduces laboratorist fatigue. Allegedly, fluorescent staining is more sensitive than Ziehl- Neelsen staining (Steingart *et al.*, 2006). Because of the rapidity of the fluorochrome method, laboratories processing large numbers of specimens should adopt this technique. A real disadvantage of the fluorochrome method is that fluorescence fades with time. For this reason, the slides must be read within 24 hours. This staining method is not often available in developing countries due to the high cost of the fluorescence microscope and, especially, that of its maintenance. The results of the smear microscopy should be reported according to an internationally agreed quantitation scale (Waard, 2007).

In this study, a comparative study of ZN staining method and auramine staining method is performed for the diagnosis of acid fast bacilli. Fluorescence microscopy is most popular nowadays as it is far easier, rapid and more sensitive technique (Cheesbrough, 2006). The technique was modified and simplified by eliminating heating step and combining the stages of

decolourisation and counter staining. When acid-fast organisms are observed on a smear, results must be quantified to be meaningful because this quantitation estimates the number of bacilli being excreted; the extent of a patient's infectiousness can be assessed for clinical and epidemiological purposes (Forbes *et al.,* 1998).

The specificity of microscopy is high, ranging between 98-99% but the sensitivity is relatively poor at 50-70% when compare with culture. The sensitivity of the acid-fast smear examination for the diagnosis of mycobacterial infection is lower than that of culture methods. The factors influencing sensitivity includes type of specimen, concentrations of mycobacteria in specimens, and staining techniques. Respiratory specimens yielded the highest smear positivity rate, followed by tissue specimens and cerebrospinal fluid. Furthermore, if more than one respiratory tract specimen is submitted to the laboratory, 96% of patients with PTB may be detected by the acid-fast smear examination (Kar *et al.*, 2003; Balows *et al.*, 1991).

Lipsky *et al.,* (1984) studied the factors affecting clinical value of AFB and reported that out of 3,207 clinical specimens submitted for mycobacterial smear and culture, Mycobacteria grew from 176 (5.5%) of the specimens, 95 (54%) of which were *M. tuberculosis*. Although the overall sensitivity of the smear was low (33%), 65% of respiratory specimens yielding *M. tuberculosis* had positive AFB smears. Furthermore, 96% of patients with PTB from whom more than one specimen was processed had at least a single positive AFB smear. Smear sensitivity correlated well with quantitative growth; 89% of specimens yielding greater than or equal to 50 colonies per slant were smear positive. Specificity of the AFB smear was high; 89% of smear-positive specimens had positive cultures. After the results from culture-negative patients known to have active TB were eliminated from the analysis, the specificity of a positive smear raised to 98.3%. When the results of all specimens from each patient were

considered in total, the AFB smear had a predictive value of greater than or equal to 96%. Shea (2009) found that a total of 72 (69.2%) of 104 specimens from 104 patients were culture positive for mycobacterium tuberculosis. The fluorochrome stain was positive for 67 of 7 culture positive specimens (sensitivity 93.1%) and negative for all 32 culture negative specimens (specificity 100%).

Mendoza and Narciso (1987) compared the light microscopy of ZN with that of fluorochrome dye for detection of AFB and reported that out of 2,182 sputum specimens 159 were found to be positive for AFB. These included sputum from 132 specimens positive for AFB both ZN and auramine staining methods and sputum from additional 27 specimens positive for AFB by auramine staining only. Mendoza and Narciso (1987) conducted a study to reassess efficiency of the conventional ZN and fluorescent AR staining techniques of direct microscopy for the detection of AFB from various clinical samples. In the study, a total of 1046 consecutive samples comprising 731 respiratory samples, 213 urine, 68 body fluid, and 34 tissues was studied and found that 8.89% AFB positivity using both the techniques (ZN and AR); whereas culture showed that 137(13.09%) *M. tuberculosis* growth. Only 67.9% of the culture positives specimens were smear positive. In the study AR was 65.4% sensitive and 96.2%specific and ZN was 50% sensitive and 97% specific.

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 FM and ZN methods, respectively. The sensitivity of the FM method was 80% and that of the ZN method 65% ($p < 0.001$). Overall agreement was 86.8%. The use of FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears. The method is economical in both time and expense and is

recommended for laboratories handling large numbers of sputum specimens.

Jain *et al.,* (2002) conducted a study to reassess efficiency of the conventional ZN and fluorescent AR staining techniques of direct microscopy for the detection of AFB from various clinical specimens. In the study, a total of 715 consecutive samples comprising 493 sputum samples, 76 cerebrospinal fluid, 66 fine needle aspirates, 30 pus samples, 5 urine sample and 45 miscellaneous body fluids was studied and found that 32.3% AFB positivity using both the techniques (ZN and AR); 42.2% in sputum and 9.9% in extra-pulmonary specimens. ZN staining showed 23.4% AFB smear positivity; 32.7% in sputum and 1.4% in extra-pulmonary specimens. AR staining showed 31.8% AFB smear positivity, 41.6% in sputum and 9.9% in extra-pulmonary specimens. Overall 208 cases were found to be positive in which ZN contributed only 164 (78.8%) cases which included 3 cases (1.4%) missed by AR. The AR found 205 (98.5%) cases and missed 3 cases; the difference in case yields was highly significant ($p<0.001$). the difference was more marked in extra pulmonary samples. In the study AR was 86.6% sensitive and ZN was 67.3% sensitive and a total of 46.4% were smear positive while 48.1% were culture positive.

Ziaee *et al.,* (2008) conducted study to compare the value of two different staining for diagnosis of acid fast bacilli. A total of 102 out of 920 study subjects had pulmonary tuberculosis, of them 68 (66.66%) patients were smear positive by either staining method while other were smear negative. The proportion of positive smears detected was 51% and 57% for the ZN and auramine phenol staining methods, respectively. The sensitivity, specificity, positive predictive value and negative predictive value were 51%, 100%, 100%, 94% and 57%, 100%, 100%, 95% for the ZN and auramine phenol staining methods, respectively.

Laifangbam *et al.,* (2009) found that out of Out of 102 patients, 44.1%, 71.6% and 70% were found positive by ZN, AO and culture respectively. AO was

found to be superior to ZN on several aspects. The difference in their case detection rates was statistically significant (2 = 24.93, $p < 0.001$). AO was also able to detect more pauci-bacillary cases than ZN. There was more agreement between culture and fluorescence microscopy (95.1%) than with ZN microscopy (69.6%). The percentage of false negative by AO staining was only 2.78% which was in sharp contrast to that of ZN (40.27%).

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

Ulukanligil *et al.,* (2000) found that 68 patients (23.1%) were diagnosed to have TB from 295 patients by culture. The ZN and Fluorescence microscopy (FM) sensitivities 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 ZN and FM sensitivities were 66% and 83% respectively. In 76 patients (25.8%) who submitted three specimens, TB positivity was determined in 20 (26.3%) of them and the sensitivities were 80% and 92% in the ZN and FM stained smears respectively.

Annam *et al.,* (2009) studied the comparative study of ZN method to auramine method in detection of acid fast bacilli in lymph node aspirates. Out of 108 aspirates, 102 were studied and remaining 6 were excluded from the study due to diagnosis of malignancy in 4.04% (4/6) and inadequate aspiration in2.02% (2/6). Among the 102 aspirates, 44.11% (45/102) were positive for AFB on conventional ZN method, 58.9% (60/102) were indicative of TB on cytology, while the smear positive increased to 81.37% (83/102)

on the modified fluorescent method. 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 AR, and 86.4% and 96.2% in out of 311 sputum samples for Ehrlich-Ziehl-Neelsen (EZN) respectively, when culture was accepted as reference method.

Singh and Parija (1998) compared the light microscopy of ZN with that of fluorochrome dye for detection of AFB and reported that out of 2600 clinically suspected patients, sputum specimens from 1,104 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. Prasanthi & Kumari (2005) also reported that fluorochrome staining was found to be more efficient (45%) when compared to ZN staining (29%) in detecting cases associated with HIV seropositivity, especially paucibacillary cases.

In a study Somoskovi *et al.*, (2001) evaluated the routine performance of four different staining methods (Kinyoun, ZN, auramine, AR) and it was found that Kinyoun cold carbol fuchsin method is inferior to both the ZN and Fluorochrome (auramine and/or AR) methods. A comparison of readings with the four different staining methods revealed a significantly higher sensitivity of AR Vs Kinyoun ($p < 0.01$), ZN ($p = 0.01$), and auramine ($p < 0.02$). The difference between auramine and Kinyoun ($p = 0.064$), and auramine and ZN ($p = 0.089$) which was close to being significant, while there was no significant difference between Kinyoun and ZN ($p = 0.73$). Similar type of research was carried out by Shrestha (2006) a total of 1365 specimens from 500 patients were analyzed. Out of which 109 patients (21.8%) were diagnosed as having TB by culture. The positive yield found with the staining techniques were 14.2% by ZN, 13.8% by Kinyoun, 14.4% by Modified Cold stain and 17.6% by Fluorochrome staining methods. With reference to culture, sensitivity of ZN, Kinyoun, MC and Fluorochrome were found to be

57.8%, 56%, 59.6%, and 71.6% respectively. The specificity in ZN, Kinyoun and Fluorochrome methods was 98% and that in MC was 98.2%. The positive predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 88.7%, 88.4%, 90.3% and 90.7% and the negative predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 89.3%, 88.9%, 89.7% and 92.5% respectively.

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 (FP) results. Data suggest that the smear is a poor screening technique in a population where the prevalence of TB is low (Boyd and Marr, 1975). But Gordin and Slutkin (1990) reported that the positive predictive value (PPV) of acid-fast microscopy was 97.9% and 100% in both low and high laboratory prevalence periods of *M. tuberculosis.*

Positive acid-fast sputum smears in culture-proven MTB was found in similar frequency in patients with and without HIV. The absence of cavitary disease did not significantly reduce the frequency of positive acid-fast smears. For patients with HIV, the likelihood of a positive smear was also independent of CD4 cell counts and drug resistance. Patients with HIV and disseminated TB had positive sputum smears in nearly all cases (Smith *et al.*, 1994).

3.9.3.4.1 Concentration of Sputum Sample

Sputum is the most common specimen received for TB diagnosis. The minimum number of bacilli needed to detect their presence in stained smears has been estimated to be 5,000-10,000 per ml of sputum. For diagnosis, the sensitivity of AFB smear staining relative to culture has been estimated to vary from 50 % to over 80 % (Garay, 2000). Several studies have been published on improving smear microscopy performance using methods that concentrate the bacilli present in the sputum specimen. The methods

consist of submitting the specimen to a liquefaction step prior to concentrating it by sedimentation or centrifugation. The smears are then performed from the sediment and stained for microscopic examination. The best known concentration procedure is the 'bleach microscopy method', in which the sputum is liquefied with sodium hypochlorite (NaOCl or household bleach), and concentrated by centrifugation before AFB staining. One study done at Tanzania showed that concentration by NaOCl increases the smear positivity rates by 15.6 (Makunde *et al.*, 2007). Similarly other many study using NaOCl concentration method showed increased sensitivity than direct microscopy (Farnia *et al.*, 2002; Matu *et al.*, 2007). This technique is inexpensive and easy to perform. In addition, NaOCl is a potent disinfectant that also kills mycobacteria, thus reducing the risk of laboratory-acquired infection but, at the same time, rendering the method unsuitable for culturing. A significant improvement in the proportion of positive AFB smear results has been reported, ranging from 7 % to 253 % (Angeby *et al.*, 2004). Other concentration methods should be used if the specimen is to be cultured. The sediment of a sputum specimen liquefied and decontaminated with sodium hydroxide- N-acetyl-L-cysteine method and concentrated by centrifugation can also be examined by AFB smear staining. An advantage of this method is that the same sediment can be cultured, in contrast to those liquefied with the NaOCl method described above. Other methods involving sputum liquefaction with different substances, and concentration either by sedimentation or centrifugation, have been proposed. The methods using dithiothreitol (Murray, 2003), sodium citrate (Bahador, 2006), chitin (Farnia *et al.*, 2004) and C (18)-carboxypropylbetaine (Scott, 2002) have been evaluated favorably for the preparation of concentrated smears. Sputum concentration methods were also reviewed (Steingart *et al.*, 2006) and compared with direct smears. The authors concluded that concentration by centrifugation and sedimentation with any of several chemical methods (including bleach) is more sensitive

than direct AFB sputum smear examination.

However, other have called for more evidence before implementing any policy change, citing concerns such as increased cost for materials and training, higher biosafety requirements and difficulty standarding techniques across sites (Ramsay *et al.*, 2006). Of equally great concern, sputum concentration carries a risk of decreased sensitivity (e.g., through destruction of bacilli during concentration) (Wilkilson *et al.*, 1997) and specificity (e.g., through contamination during additional transfer steps) (Ramos *et al.*, 1999; Angeby *et al.*, 2004).

3.8.3.4.2. Limitations of Sputum Smear Microscopy

Although relatively rapid, simple, and inexpensive, sputum microscopy suffers from following drawbacks.

1. The acid fast stain is non specific. Slow growing mycobactria are consistently acid fast. Microorganisms other than mycobacteria that demonstrate various degree of acid fastness include *Nocardia, Rhodococcus*, *Legionella micdadei*, Cysts of *Cryptosporidium* spp. and *Cyclospora* spp.
2. The acid fast smear is insensitive. Sensitivities ranging from 22 to 81% have been reported. A negative smear does not rule tuberculosis because low bacterial load cannot be detected.
3. Though some research reported the increased sensitivity of microscopy (Shea *et al.*, 2009), Many data suggest that the sputum smear microscopy is a poor screening technique in a population where the prevalence of TB is low and where there is increasing burden of HIV and AIDS (Prasanthi and Kumari, 2005; Boyd and Marr, 1975).
4. Since heat fixing and staining may not kill all the

mycobacteria, discard slides in a sharps receptacle and wear gloves.

Many variables influence the results of microscopy which includes; the prevalence and severity of the disease, the quality of specimen collection, the number of mycobacterium present in the specimen, the method of processing (direct or concentrated), the staining technique, and the quality of the examination (microscope operator expertise, time spent for smear examination, etc.) (Somoskovi *et al.*, 2001). A large work load may also influence the sensitivity. False negativity due to fatigue may also contribute to decreased sensitivity (Parekh and Kar, 2003).

The short comings of AFB microscopy can seriously limit both the extent and quality of its application, and ultimately show its impact on TB control. It requires equipment that is difficult to maintain in field settings, yield results depend upon the studious attention of a trained and motivated technician, and it is notoriously insensitive especially in controlled programmes. Besides this, smear examination requires sputum collection, smearing, drying, staining and examination; delays in reporting that may lead to the substantial difficulties in case holding. The need for duplicate or triplicate sputum examination further compounds the problem (Perkins, 2000).

It is recommended that a negative result should only be reported following the examination of at least 100 (in low-income countries) and preferably 300 (in industrialized countries) microscopic immersion view fields (or equivalent fluorescent view fields). Therefore, when microscopy is performed correctly, it can be time-consuming and laborious (Somoskovi *et al.***,** 2001).

Negative results and slow reporting ultimately may erode patient faith in the services of the laboratory and of the health system. And again, a negative smear result does not exclude the diagnosis of tuberculosis, as about 55% of PTB cases worldwide, harbor lower bacillary load, so that the sputum is

negative upon microscopic observation (Parekh and Kar, 2003).

3.8.3.4.3 Quality Control of AFB Smears Microscopy

Patients with infectious TB may remain undetected due to unreliable laboratory diagnosis. In addition, errors in AFB smear microscopy reading can result in patients being put on treatment without having the disease. Therefore, quality assurance of AFB sputum smear microscopy is essential, and the quality of laboratory services should be considered a high priority of the National TB Control Programmes. These quality assurance programs are based on systematic monitoring of working practices, technical procedures, equipment and materials, including quality of stains, site evaluation of laboratory/quality improvement and also training, when needed. When the patient is thoroughly instructed on sputum sampling, the microscopic diagnosis of TB improves substantially (Alisjahbana, 2005). Poor quality of the sputum specimen often results in AFB smear microscopy negative results (Waard, 2007). Registering the quality of the sputum specimens received at the laboratory could help to improve sputum sampling. Satisfactory quality implies the presence of mucoid or mucopurulent material and a volume of 3-5 ml, although smaller volumes are acceptable if the consistency is adequate. If a relatively high percentage of the specimens received are saliva, the laboratory should report this to the medical staff, and instructions should be given to nurses and physicians on how to improve the quality of sputum sampling.

3.8.3.5 Conventional Culture Method

Culture is considered a gold standard in clinical bacteriology. It entails the cultivation of clinical specimens on synthetic media for the purpose of isolating the causative agent of a disease condition. Mycobacterial culture is more sensitive than smear microscopy as it is able to detect as few as 10 bacilli per ml of digested concentrated clinical specimen (Grange, 1998). Cultivation of MTB complex group (*M. tuberculosis*, *M. africanum,*

M. bovis, M. microti) is difficult and time consuming, requiring 3 – 8 weeks for primary isolation from clinical samples and 4–6 weeks for drug susceptibility tests. Cultures of mycobacterial species are however needful for performance of biochemical identification tests, drug susceptibility test and specific molecular methods requiring the use of genomic DNA (eg, Restriction fragment length polymorphism; RFLP). Various mycobacteriological culture media are available. These are categorized as; egg-potato based, egg-based (Lowenstein -Jensen's) and agar based (Middle Brook 7H-10, Middlebrook 7H-11 and Dubois Oleic albumin) media.

Culture media may be inoculated directly with centrifuged deposits of fluids and homogenized biopsies if it is highly unlikely that they contain non acid fast microorganism but most other specimens, especially sputum and urine, must first be decontaminated. The most widely used method is Petroff method, where sputum is mixed well with 4% sodium hydroxide for 15-30 minutes, neutralized with potassium dihydrogen orthophosphate and centrifuged. The deposit is used to inoculate on media (Grange, 1998). Similarly 2% NaOH – N-acetyl-L-cysteine method is also used for decontamination of sputum (Frobes *et al.*, 2002; Grange, 1998).

3.8.3.6 Automated Methods (Radiometric and Non Radiometric)

Automated methods are designed to yield results of primary cultures and drug susceptibility tests within the shortest possible period (4- 21 days). Their ability to combine efficiency and reproducibility overcomes most of the limitations of the conventional methods. Automated machines are commercially available from different manufacturers. These include; the BACTEC 460 TB System (TB BACTEC), a radiometric procedure which measures $14C0_2$ released during metabolism of 14C-fatty acid substrate by growing bacteria. The amount of $14C0_2$ released is expressed as growth index (GI) on a scale of 0 to 999. In the presence of an antimicrobial agent, inhibition of daily GI is considered as susceptibility of test

organism to the drug (Sidiqi *et al.*, 1981). The handling and disposal of radioactive medium in this technique is however considered a disadvantage. The mycobacteria growth indicator tube (MGIT) 960 (Becton Dickinson USA) uses a Middlebrook 7H9 broth in 7 ml plastic tube and a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. The procedure involves initial, fluorescence quenching by presence of dissolved oxygen in the broth. Subsequently, growing mycobacterial cells in the inoculated tubes consume the oxygen, producing fluorescence under 365nm UV illumination. The system continuously monitors the tubes to detect increase in fluorescence and automatically determines and interprets results. When used for drug susceptibility test, a set of drug containing and drug free (growth control) media are inoculated with the test strain. Comparison of records in the test and control tubes are automatically done and reported as susceptible or resistant. MB/BacT ALERT (BioMerieux, Durham) is a fully automated and non radiometric system that utilizes a bottle containing a colorimetric sensor embedded in its bottom. Carbon dioxide produced by microbial metabolism causes reduction in pH of medium and changes the sensor color from dark green to yellow. The color change is continuously monitored and promptly reported by the instrument. Reports of various studies have indicated the efficiency of the different machines (Adjers-Koskelat, 2003), particularly in the shorter turn out time (4-21 days) compared to that of the conventional culture method (3- 8 weeks). Their application may however be limited to research and reference laboratories due to the high cost of procuring the equipment and regular consumables.

3.8.3.7 Serological Tests

Several studies based on the detection of antibodies to a variety of mycobacterial antigens of bacteria derived antigens have been carried out but none has proved reliable so far. Lack of sensitivity and specificity are the main problems encountered when using immunological techniques.

However, their use is becoming increasely important in the diagnosis of child TB. However several research have been attempted to increases the sensitivity and specificity of serological kits. Enwuru *et al.*, (2004) performed the comparative study on specific and early detection of pulmonary mycobacteria using serological pathozyme EIA kits with reference to culture found that sensitivity of the method is improved up to 88% by using combination of IgA, IgG and IgM antibodies whereas specificity is 91%. Cytokines based assays to determine the relationship between tuberculin skin test response and cytokines profile have shown encouraging results, thus allowing epidemiological studies on the immunity to TB in humans (Guithi *et al.*, 2002).

3.8.3.8 Tuberculin Test

The tuberculin test is used to determine the annual infection rate of tuberculosis in the community, to assess the effectiveness of control measures, to indicate those requiring preventive therapy and to aid diagnosis. It is widely used as diagnostic test, although it usefulness is limited by its failure to distinguished active disease from quiescent infection and past BCG vaccination, especially in USA where BCG is not used for vaccination purpose (Grange, 1998).

3.8.3.9 Molecular Methods

Molecular methods are genetic procedures that make use of genetic materials (DNA or RNA) to detect specific proteins or genes of the test organism using specific probes or short stranded oligonucleotides (primers) complementary to the test DNA strand. The high degree of DNA polymorphism, repetitive DNA sequences and presence of insertion sequences (IS) characteristic of MTB strains have been used as basis for the study of strain-strain relatedness/diversity of MTB. Primer sequences of specific regions in the MTB genome have been done and cloned for use in detection identification and typing from clinical samples and cultures (Yang, 2003). Insertion sequence 6110 polymerase chain

reaction based diagnostic method The IS6110 PCR technique is useful in the rapid detection of MTB complex strains in clinical specimens from naturally sterile anatomical sites with minimal bacillary load, often undetectable by the conventional methods. E.g. CSF, pleural effusion, joint and marrow taps gastric washings. The method is cost-effective and simple to perform. It is specific, sensitive, reproducible and able to generate results within hours (Ani, 2008). The target DNA is PCR amplified using IS6110 primers sequences. The amplified product is electrophoresed using 2% agarose and observed by UV illumination for DNA band of 123 base pair. However some studies indicated a relatively low PCR sensitivity. As demonstrated by Cegielsi *et al.,* (1997), the sensitivity of PCR with pericardial fluid for the detection of MTB was poor in compare to conventional culture method. In their study tuberculosis was correctly diagnosed by culture is 93% whereas by PCR in 81%. Also, PCR gave one false positive result for a patient. Likewise, the main drawback of the PCR diagnostic approach is the danger of cross contamination of specimens with DNA. PCR is so sensitive that small contaminating amounts of DNA can give a positive signal (Salyers, 2002).

3.8.3.10 Molecular Epidemiology/Genotyping

Molecular epidemiology has been defined as the integration of molecular techniques to track specific strains of pathogen with conventional epidemiological approaches to understanding the distribution of disease in populations (Yang, 2003). Molecular genotyping of MTB strains is useful in outbreak investigations, comparison of isolates from different laboratories and geographical regions and contact tracing (Daley, 1992). The short repetitive DNA sequences and the insertion sequences characteristic of MTB strains have been used as basis for designing specific probes used in different techniques (Yang, 2003). Studies on the use of different methods such as the Restriction fragment length polymorphism (Van, 1993), spoligotyping, MIRU VNTR (Mycobacterial Interspersed Repetitive

Units- Variable Number of Tandem Repeats) and other PCR based techniques have been reported (Heym *et al.*, 1994). The goal ultimately is to have a general understanding of regional and global transmission of MTB isolates and for strain tracking especially those showing peculiar or unusual characteristics.

3.8.3.11 Novel Diagnostic Tests

TB diagnostics is a field of intense research and several new avenues are being pursued. **Mycobacteriophage assays** are promising novel diagnostic techniques where clinical specimens are incubated overnight with a mycobacteria-specific phage (phages are viruses that parasitise bacteria). The next morning a virucidal agent is added, neutralizing all free phage virions, so that only phages that have entered and infected mycobacteria in the specimen survive. The specimen is then plated onto a Petri dish covered with a lawn of rapidly growing mycobacteria and is incubated for 24 - 48 hours. If mycobacteria were present in the original specimen the phage will be released into the Petri dish culture and destroy areas of the mycobacterial lawn.

The interferon assay tests the *in vitro* response of patients' blood mononuclear cells against purified protein derivatives of *M. tuberculosis*. The technique is promising, but cannot distinguish between latent infection and active disease, and may not perform well in patients who are severely immunocompromised from HIV infection.

Proteomic systems hold the most promise for the future. This experimental technique concentrates and fractionates secreted mycobacterial proteins, potentially allowing these proteins to be identified in the body fluids of patients with active TB.

3.8.3.12 Drug Resistant Tuberculosis and Drug Susceptibility Test (DST)

Drug resistance, by definition, is a temporary/permanent capacity of the organisms and their progeny to remain viable or to multiply in presence of concentration of the drug that would normally destroy/inhibit the growth of

other similar cells. So, drug resistant TB is a case of TB (usually PTB) excreting bacilli resistant to one or more anti-tubercular drugs.

3.8.3.12.1 Multi-Drug Resistant TB (MDR-TB)

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

People who have spent time with someone sick with MDR TB disease can become infected with TB bacteria that are resistant to several drugs. Historically, MDR TB has spread gradually with alarming rates seen in 2001 in India, China, UK, Russia, Peru, Spain and Puerto Rico (WHO, 2007). The magnitude of anti-TB drug resistance is not well documented in the South East Asian Region. The initial drug resistance rate in Nepal is not so high comparing with that of other countries. In 1992, the primary drug resistance rate was 6.3%, 9.5% for INH and SM respectively. First surveillance of TB drug resistance survey showed initial MDR 3.7% and acquired 12% (Shrestha, 1996). Gharti Chettri, 2002; reported that 8.57% of the isolates had primary MDR-TB while 100% of the isolates had acquired MDR-TB. Bhattarai, 2003; reported 4.61 % and 5% of the total isolates had primary and acquired MDR-TB respectively. Similarly the study carried out by Subba, 2007 reported Multi drug resistance (MDR) in untreated TB patients were found in 22.22 % and in treated TB patients was 37.20 %.

3.8.3.12.2 Extensive Drug-Resistant TB (XDR-TB)

XDR-TB or extensive drug-resistant TB, is currently defined as resistance to the two most potent anti-TB drugs-INH and RFP, and resistance to at least two of the six classes of second-line drugs i.e. aminoglycosides, polypeptides, fluoroqunolones, thioamides, cycloserine and para-aminosalicylic acid. These strains leave patients without treatment options that meet international standards and are therefore virtually untreatable (WHO, 2007). By the end of 2008, 55 countries (including Nepal) and territories had reported at least one case of XDR-TB, including five that reported cases for the first time in 2007 (Colombia, Oman, Qatar, the United Arab Emirates and Uzbekistan) (WHO, 2009).

3.8.3.12.3 Drug Susceptibility Test

Performance of drug susceptibility test is useful for the diagnosis of drug resistant and multi drug resistant MTB isolates. Several techniques based on phenotypic and genotypic principles have been developed for determination of DST of MTB. Three (phenotypic) methods are generally used in routine practice: The absolute concentration method, resistance ratio method and proportion method. The tests can be performed by the direct inoculation suspension of pure cultures obtained from growth on synthetic media (Lowenstein-Jensen, 7H10 or 7H11 agar). The automated methods already described are comparatively more advantageous in producing results within days to a few weeks (Ani, 2008).

Genotypic methods are useful in the understanding of the molecular basis of drug resistance. The methods which include; DNA sequencing (Heym *et al.*, 1994), Real-Time PCR (Torres *et al.*, 2000), DNA Microarrays (Troesch *et al.*, 1999), are used for rapid detection of mutations associated with specific and multi drug resistant tuberculosis.

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials and Chemicals Used

A list of materials, chemicals, equipments and reagents required for this study is presented in Appendix No 1.

4.2 Methodology

This study was conducted at Microbiology Laboratory of Bir Hospital, Kathmandu, Nepal. The patients refer for AFB staining in this hospital, were selected for the study. Samples were collected from patients visiting the Bir hospital with symptoms suspected of tuberculosis (pulmonary and extra pulmonary) from out patients department as well as in patients department like: medical wards, tropical wards, to Microbiology Laboratory were included. Patients with insufficient sample quantity and lack of patient details were excluded from the study.

4.2.1 Case Definition

Once acid fast bacilli (>2 bacilli) were identified on any smear examination, the examinee was identified as a 'positive case'.

4.2.2 Sample Collection and Processing

A total of 2,592 different specimens from 1,019 patients suspected of tuberculosis (pulmonary and extra pulmonary) were taken. In case of PTB, early morning purulent sputum samples were collected. The patients were provided with sterile, leak proof, disposable and appropriately labeled wide mouthed container. The patients were instructed to provide deep- cough sputum, not saliva or nasal secretions. In case of extra pulmonary tuberculosis, specimens like urine (whole morning), ascitic fluid, pus, CSF, etc were collected in leak proof, disposable and well labeled container.

A Semi structured Proforma was used to collect information of patients (Appendix No. 2). For staining each sample, two individual slides were

prepared for ZN and Fluorochrome stains. Remaining sediments was disposed after autoclaving.

4.2.3 Concentration of Samples

Early morning purulent sputum sample were collected for smear preparation in case of pulmonary tuberculosis. Extra pulmonary samples, like different body fluid however, were concentrated by conventional centrifugation method. The sediments were used for preparation of smear. In case of urine specimen, the container on which sample were collected, were left for whole night and upper part were drawn off and sediments were taken for centrifugation. After centrifugation, the deposits were taken for smear preparation. The remaining sediments were disposed after autoclaving.

4.2.4 Preparation of Smears

Early morning purulent sputum was recommended for diagnosis of pulmonary tuberculosis. Smear or sample preparation was done in UV chamber. Sputum and other pulmonary samples were smeared evenly with an uneven end of broom stick on the two slides each labeled for staining techniques respectively, the smear size being 2 cm x 3 cm and it should not too thick. The smear was air dried in UV chamber before being fixed. Heat fixation was done by passing the air dried smear over Bunsen burner. The slides were then placed in serial order on the staining rack with the smeared slides facing upward ensuring slides do not touch each other.

4.2.5 Staining Methods

The staining procedure was same for both pulmonary and extra pulmonary specimens except urine specimens where 3% acid alcohol was used as decolorizing agent. As this decolorizing agent content both acid and alcohol as decolorizing agent urine specimens which is likely to contain saprophytic mycobacterium only *M. tuberculosis* retains this decolorizing procedure.

4.2.5.1 Ziehl-Neelsen Staining

After smear preparation and heat fixation, the slides were placed on staining rack with the smeared slides

facing upward ensuring slides do not touch each other. Then, 1% carbol fuchsin was poured to cover the entire surface of the slides. The slides were heated underneath until vapour start rising. The slides were allowed to stand for about 10 minutes. The slides were then rinsed with tap water and excess water was drained off. The slides were decolorized with 20% sulphuric acid for about 10 minutes. The slides were rinsed thoroughly with tap water and excess water was drained off. The slides were flooded with 0.3 % methylene blue solution and were let to stand for 1-2 minute. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry. The slides were examined under microscope in1000x oil immersion.

4.2.5.2 Fluorochrome Staining

The slides were placed on staining rack as same as ZN staining technique. Then the entire smear was flooded with 0.1% auramine phenol and was allowed to stain for about 10 minutes, ensuring that staining solution remains on smears. The slides were rinsed with distilled water and excess water was drained off. The slides were decolorized with 20% sulphuric acid for about 10 minutes. The slides were rinsed with distilled water and drained off. The slides were flooded with 0.3% methylene blue and allowed to counter stain for about 2 minutes. The slides were rinsed with distilled water and drained off. The slides were allowed to air dry. The slides were examined under fluorescence microscope at low power (40×) as soon as possible after staining.

4.2.6 Precautions

As tuberculosis is highly contagious disease avoidance of aerosol formation prevents the infection of laboratory personnel. All the specimens were processed in biosafety cabinet. Generally preparation of smear and drying of prepared smear was processed in biosafety cabinet.

The potential contamination rate of smear preparation was though less, contamination can occur if negligence of smear preparation was performed.

Thus risk for contamination had to be strictly avoided. As a general rule, preparation of smear was done by separate bamboo stick, during staining slides should be apart from each other. It is also recommended to clean the objectives after observation of positive slides. The working surface in the hood was bleached with 5% bleach and UV-radiated prior to smear preparation.

Before every new smear preparation specimens to be prepared were placed in UV radiated wood for at least 15 min. This was necessary, because specimens might be leaked during sample collection and was likely to contaminate other slides during preparation.

4.2.7 Quality Control

Positive and negative control slides were included with each staining batch for internal quality control of the staining methods. Blinded reading of the slides was done by experienced microscopist. In addition all smears were independently reported by same microscopist and results were tallied before reporting. All reagent preparation was done by standards procedure (Appendix-3). All reagents were prepared by using distilled water to avoid false positive results (Collins et al, 1997).

4.2.8 Recording and Interpretation of Microscopy Results

In recording and reporting of microscopic results, the following reporting scale was used for ZN stain, as per the guidelines given by IUATLD.

Number of bacilli in smear
Results reported

No AFB per 300 oil immersion fields
Negative

1-9 AFB per 100 oil immersion fields
Record the exact number

10-99 AFB per 100 oil immersion fields
1+

1-10 AFB per oil immersion field up to 50 fields 2+

>10 AFB per oil immersion field up to 20 fields 3+

The number of AFB found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease. The number of bacilli observed by Fluorescence microscopy was divided by 4 to correlate with the results obtained by ZN microscopy.

4.3 Statistical Analysis

All the data were collected and analyzed by using Microsoft excel. Statistical analysis was performed by using 2 – test at 5% level of significance.

Chapter V

5. Results

5.1 AFB Finding by ZN Microscopy and Fluorescence Microscopy

During the period from December to early March (2009-2010), a total of 2,592 different specimens from 1,019 patients (comprising pulmonary samples 2,492 from 925 and extra pulmonary samples 100 from 94 patients) suspected of tuberculosis received in Microbiology Laboratory were stained with both ZN stains and auramine-phenol (Fluorescence) stains. Altogether 162 (6.25%) specimens were detected as AFB positive by either of the method. However, 160 (6.17%) specimens were found to be positive by Fluorescence microscopy while only 140 (5.4%) specimens showed positive by ZN microscopy method (Table 1).

Table 1: ZN Microscopy and Fluorescence Microscopy Finding

	Fluorescence Microscopy		
ZN Microscopy	Positive	Negative	Total
Positive	138	2	140
Negative	22	2430	2452
Total	160	2432	2592

Comparison of Fluorescence and ZN microscopy

Both techniques were in agreement that 2430 (93.75%) were negative smears. Only two smears (0.077%) found to be AFB positive by ZN microscopy showed negative result by Fluorescence microscopy and both of these smears contain low density bacilli. Twenty AFB positive smears (0.77%) picked up by Fluorescence microscopy was found to be negative by ZN microscopy. These twenty slides found to be negative by ZN microscopy were over stained by ZN staining method. Upon reexamination, all these 20 slides were also found positive. Thus, this reexamined result gave a positive yield of 162 out of 2,592 (6.25%) by Z-N microscopy against the 160 out of 2,592 (6.17%) by Fluorescence microscopy.

The higher proportion of positive results seen in fluorescence microscopy than in ZN microscopy was found statistically significant difference (2= 2164.69, p< 0.05).

5.2 AFB Finding of Sputum Sample (Pulmonary Cases)

A total of 2,492 sputum specimens from 925 suspected patients were analyzed during the study. Out of 2,492 sputum smears, 155 (6.22%) smears from 63 patients showed AFB positive by either method. Fluorescence microscopy gave 153 (6.14%) positive smears from 61 patients while ZN microscopy gave only 133 (5.34%) positive smears from 56 patients (Appendix 6).

Table 2: Sputum Smears Finding by ZN and Fluorescence Microscopy*

Total number of sputum sample	**ZN microscopy**		**Fluorescence microscopy**	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
2,492	133 (5.34)	2,359 (94.66)	153 (6.14)	2,339 (93.86)

* Fluorescence microscopy gave 20 (0.8%) positive smears which were found to be negative by ZN microscopy.

Only 2 (0.08%) smears reported AFB positive by ZN method were negative by Fluorescence microscopy (Appendix 6).

Thus, the higher proportion of positive results seen in fluorescence microscopy than in ZN microscopy was found statistically significant difference (2= 2062.71, $p < 0.05$) (Appendix 4).

5.2.1 Demographic Characteristics of Pulmonary Positive Cases

Of the 925 suspects 63(6.81%) were cases of pulmonary tuberculosis by AFB (either

ZN or Fluorescence) staining method. The positive cases was lowest (no case) among children age groups (< 11 years). The largest proportion of cases was found in those aged 10-50 years (9.06%) (Table 3).

Table 3: Agewise Distribution of AFB Positive Cases among Pulmonary Tuberculosis

Age (years)	Number of	Positiv e	Percenta ge (%)

	Suspected	Cases	
10	9	0	0
11-20	109	11	10.09
21-30	182	22	12.09
31-40	123	3	2.44
41-50	116	12	10.34
51-60	145	9	6.21
61-70	124	2	1.61
>70	91	4	4.4
Unknown	26	0	0
Total	925	63	6.81

In sexwise distribution, largest proportions of suspects were males (60.95%). Among positive cases 44/63 (69.84%) were male against 19/63 (30.16%) female (Table 4).

Table 4: Sexwise Distribution of AFB Positive Cases among Pulmonary Tuberculosis

Sex	Suspects	Positive Cases	Percentage (%)
Female	361	19	5.26
Male	564	44	7.8
Total	925	63	6.81

5.3 AFB Finding of Extra Pulmonary Samples

Altogether 100 different extra pulmonary samples from 94 patients were analyzed that consists, Pleural fluid (29), Ascitic fluid (26), Pus (10), CSF (11), Urine (11), Peritoneal fluid (2), Synovial fluid (2), Hydrolic fluid (1) and miscellaneous aspirated fluid (8) (Table 5).

Table 5: Distribution of Different Extra Pulmonary Samples

Specimens	Number of Suspects	Number of specimens	Positive	
			Suspects	Specimens
Pleural fluid	29	29	0	0
Ascitic fluid	26	26	0	0
Pus	10	10	0	0
CSF	11	11	0	0
Urine *	5	11	3	6
Peritone	2	2	0	0

al fluid				
Synovial fluid	2	2	0	0
Hydrolic fluid	1	1	0	0
Miscellaneous fluid**	8	8	1	1
Total	94	100	4	7

*= 11 urine specimens were obtained from 5 patients as (9 specimens from 3 patients and 2 specimens from 2 patients) and 6 positive specimens from 3 patients were obtained as (3 specimens from 1 patient, 2 specimens from1 patients and 1 specimen from1 patient). **= miscellaneous fluid contains different aspirated fluid and the positive smear (1) was found in elbow aspirate

Out of these 100 different samples only 7 specimens (1 elbow aspirate and 6 urine samples) from 4 patients gave positive result (Table 5).

Altogether 7 specimens were found to be AFB positive by both ZN microscopy and Fluorescence microscopy. There was no difference between ZN and Fluorescence microscopy in this study group samples (Table 6).

Table 6: Extra Pulmonary Samples Finding by ZN and Fluorescence Microscopy

Total number of samples	**ZN microscopy**	
	Positive (%)	Negative (%
100	7 (7)	93 (93)

5.3.1 Demographic Characteristics of Extra Pulmonary Positive Cases

Of the 94 suspects 4 (4.25%) were cases of extra pulmonary tuberculosis by AFB (either ZN or Fluorescence) staining method. The positive cases was lowest (no case) among age groups (< 20 years) and the largest proportion of positive cases was found in those aged 21-40 years (4.25%) (Table7).

Table 7: Agewise Distribution of AFB Positive Cases among Extra Pulmonary Tuberculosis

Age	Number	Positiv	Percentag

(years)	of Suspecte d	e Cases	e (%)
10	2	0	0
11-20	8	0	0
21-30	28	1	3.57
31-40	21	3	14.28
41-50	11	0	0
51-60	12	0	0
61-70	7	0	0
>70	5	0	0
Total	94	4	4.25

In sexwise distribution largest proportions of suspects were males 55/94(58.5%). Among positive cases 2/4 (50%) were male and 2/4 (50%) were female (Table 8).

Table 8: Sexwise Distribution of AFB Positive Cases among Extra Pulmonary Tuberculosis

Sex	Suspect s	Positiv e Cases	Percentag e (%)
Femal e	39	2	5.13
Male	55	2	3.64
Total	94	4	4.25

Chapter VI

6.1 Discussion

In developing countries, the diagnosis of tuberculosis is mainly done by chest X- ray, direct smear microscopy and tuberculin test. Though mycobacterium culture and anti-tuberculosis drug susceptibility tests are gold standard and should be done routinely were available only at central level. This is also the case in Nepal, where culture and DST was done only at national tuberculosis centre, Thimi and German Nepal tuberculosis project, Kalimati.

According to Harries *et al.*, (1998), the sputum positive cases are most infectious and contribute substantially to transmission of disease. But as per observations of Behr *et al.*, (1999), though tuberculosis patients with sputum smears negative are less infectious, both theoretical and empirical evidence suggests that they can still transmit tuberculosis. Thus, proper identification of cases is the pillar of tuberculosis control programme (Haubner *et al.*, 1993). Diagnosis of tuberculosis and monitoring of treatment progress rely heavily on bacteriological examination of clinical specimens. Despite recent advances in Mycobacteriology, early laboratory diagnosis of tuberculosis still relies on the examination of stained smears. Microscopy of sputum smears makes a particularly important contribution since the technique is simple, inexpensive and detects those cases of pulmonary tuberculosis for maintaining the tuberculosis. Currently no other diagnostic tool is available which could be implemented affordably (WHO 2007).

In this study, sputum from additional 20 specimens was positive by Fluorescence microscopy (85.8% by ZN, 98.7% by FM). This shows that fluorescence microscopy of sputum smears in comparison to that of ZN microscopy is better (2=2062.71, $p < 0.05$) for demonstration of acid fast bacilli. The slides which were reported as AFB negative by ZN microscopy, most of them (10) were recorded as low density bacilli, 4 were cases of follow up and rest were recorded as 1+ by

Fluorescence microscopy. Fluorescence microscopy has added advantage of allowing a large number of sputum specimen to be examined in a given time as low power is used for examination. This study correlates with the results of Githui *et al.*, (65% by ZN, 42.5% by FM), Ulukanligil *et al.*, (67.6% by ZN, 85.2% by FM) and Ziaee *et al.*, (51% by ZN, 57% by FM). The use of Fluorochrome stain greatly improves the diagnostic value of the sputum smears especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears.

In this study, however there was no difference between ZN and Fluorescence microscopy was observed for diagnosis of extra pulmonary samples. This result contradicts many other studies (Jain, 2002). This might be due to the presence of enough density of bacilli in the positive samples of extra pulmonary tuberculosis suspected specimens during the study period.

Though WHO recommended three sputum sample (one of which is early morning) for diagnosis of pulmonary tuberculosis, this is not always possible in laboratory. This is due to lack of awareness among people, need of single specimen in follow up case and sometimes even medical officer requests for analysis of single sputum specimen. During the study, altogether 2,592 specimens from 1,019 patients suspected of tuberculosis (pulmonary (925) and extra pulmonary (94) patients) were received. Among pulmonary tuberculosis suspects altogether 2,492 sputum specimens from 925 patients were received in laboratory. Likewise, in case of extra pulmonary tuberculosis total 100 different specimens from 94 patients were received.

Establishing the validity of results obtained with fluorescence microscopy by culture of pathogenic species of the mycobacterium tuberculosis complex as gold standard is not feasible in many low income laboratories. This was also the case in this study. Though the gold standard for the diagnosis of tuberculosis could not be processed in this study, the result showed that the

result obtained by Fluorescence microscopy was higher than ZN microscopy. In this study, 20 more AFB positive smears were detected by fluorescence microscopy than ZN microscopy. The result obtained in this study was comparable to other different study. The comparative study of ZN and fluorescence microscopy for detection of AFB in different clinical specimens done by Jain (2002) showed that sensitivity of fluorescence staining was 86.6% where as ZN microscopy was 67.3% sensitive, when culture was taken as reference. Similarly Mendoza *et al.*, (1986), Ulukanligil *et al.*, (2000), Shrestha (2006), Ziaee *et al.*, (2008), Laifanbam *et al.*, (2009), Singh *et al.*, (1998) and Prasanthi *et al.*, (2005) all were reported that there was increased sensitivity from 4 – 30% by fluorescence microscopy to ZN microscopy.

In this study, because of lack of culture facilities though how many false positive results were reported was not possible but all ZN negative smears which were positive by fluorescence microscopy were restained by ZN staining technique and reexamined for confirmation. All of the AFB positive slides by fluorescence microscopy also showed AFB positive after restained.

In this study, the prevalence of pulmonary tuberculosis was high in economically productive age group 11-50 years (9.06%) and among positives, in males (69.84%) as compared to females (30.16%). TB was not diagnosed in suspected cases below 11 years. On the basis of other age wise distribution, no significant difference was seen in TB cases. A study conducted by Shrestha (2006) has also reported increased incidence of tuberculosis in 15-34 years and in positive cases, 66.97% of males. Similar results were reported by Bhatt in 2009, Sapkota in 2008; in Nepal and also by Mabaera *et al.*, in 2006, and Finch in 1997, in other country. This does not however reflect an increase in the occurrence of disease in males, since the attendance of females to OPD is lower (39.03%) than males (60.97%). Also, the high suspected cases of TB in males is might be due to movement of males in different TB endemic and

crowded areas (where diseased person might be present) than females. The highest positivity among 11-50 ages group might be as a result of the fact that individual of this age group in their life tend to be more active and are more likely to interact with other people than the elderly and the young one.

Likewise, in case of extra pulmonary tuberculosis, high prevalence (4.25%) of disease was observed in 21-40 age groups and among positive, in males and in females the prevalence rate was found to be similar (50% for each group).

During study, out of 64 follow up patients 4 smear positive cases were found. The finding of AFB during treatment is not necessarily significant, as bacilli found on microscopic examination may be non viable (Morris *et al.*, 1996). However, AFB found during treatment of an initially sputum smear positive patients are highly likely to be tubercle bacilli, either dead or alive. Thus, finding AFB in a higher frequency microscopy would indicate a higher sensitivity rather than a lower specificity of the fluorescence microscopy technique in identifying tubercle bacilli. Thus the microscopy using the fluorescence microscopy was found to be useful and effective to ZN microscopy.

6.2 Conclusion

This study shows the use of Fluorescence microscopy greatly improves the diagnostic yield for demonstration of AFB in various clinical samples, particularly in patients with a low density of bacilli who are likely to be missed on ZN stained smears.

In case of single specimens for AFB staining, fluorescence staining is method of choice. Also, Fluorescence microscopy is easier to use, quicker and cheaper especially where large number of specimens (>25 sample/day) is processed. Thus the fluorescence microscopy is recommended for routine use in a laboratory. This study has been also exemplary for other laboratories, where fluorescence microscopy for

diagnosis of acid fast bacilli is planned to be introduced.

Chapter VII

7.1 Summary

The major findings of the study are summarized as follows:

1. A total of 2,592 different specimens (pulmonary 2492, extra pulmonary 100) from 1,019 patient suspects visiting Bir Hospital, were selected for the study. Among 2,592 specimens, 162 AFB positive smears (6.67%) were diagnosed by either staining methods (ZN and/or Fluorescence) method).
2. Out of 162 AFB positive cases, 160 were positive by the Fluorescence microscopy and 140 by Z-N microscopy. Twenty more positive smears (12.34%) were picked up by Fluorescence microscopy which was found to be negative by the Z-N method.
3. In sputum smears, altogether 155 AFB positive slides were found by either of the method. Out of 155 positive cases, fluorescence microscopy gave 153 positive while ZN microscopy gave only 133 of them. The difference in result obtained by ZN and Fluorescence microscopy was found to be statistically significant ($p < 0.05$).
4. However, in case of extra pulmonary samples, both ZN and Fluorescence microscopy gave 7 AFB positive slides out of 100 different samples. There was no difference in result obtained in this group of samples.
5. In case of pulmonary tuberculosis, the AFB positive cases was lowest (no case) among children age groups (< 11 years). The largest proportion of cases was found in those aged 11-50 years (9.06%) and among positive cases high prevalence rate was found in male (69.84%).
6. In case of extra pulmonary tuberculosis, high prevalence of disease was observed in 21-40 age groups (4.25%) and among positive, in males and in females

the prevalence rate was found to be similar (50% for each group).

7.2 Recommendations

The study was carried out for laboratory diagnosis of acid fast bacilli in different clinical specimen by ZN and Fluorescence Microscopy and following points can be made for the recommendations:

1. The study showed Fluorescence microscopy has greater sensitivity than ZN microscopy particularly in the case of single specimen.
2. Further comparison of Fluorescence microscopy with culture method is recommended.

Appendix-1

Materials and chemicals used

Reagents:

Carbol fuchsin

Phenol

Ethanol

Absolute alcohol

Methylene blue

Auramine

Concentrated HCL

Sulphuric acid

Materials:

Lysol

Methylated spirit

Immersion oil

Gloves

Slide box

Bunsen burner

Diamond pen

Filter paper

Slides

Equipments:

Oven

Binocular microscope

Bio-safety cabinet

Fluorescence microscope

Refrigerator

Distilled water plant

Appendix-2

Proforma

Lab No: Date:

S. No:

Identification of patient:

Name: Age: Sex: M/F

Clinical History:

Investigation:

Report:

Appendix-3

Staining reagent preparation:

Staining reagents

Ziehl-Neelsen stain

i) Ziehl-Neelsen carbol fuchsin

Alcoholic basic fuchsin (saturated solution)

Ingredients	composition
Basic fuchsin	1g
Ethyl alcohol, 95%	10ml

Phenol, 5%

Ingredients	composition
Phenol	5g
Distilled water	100ml

ii) Decolorizing agents

20% Sulphuric cid

Ingredients	composition
Sulphuric acid	20ml
Distilled water	80ml

3% acid alcohol

Ingredients	composition
Concentrated HCL	3ml
Absolute alcohol	100ml

iii) Counter stain

Ingredients	composition
Methylene blue	0.3g
Distilled water	100ml

Fluorochrome stain

i) Auramine O Fluorochrome stain

Alcoholic Auramine solution

Ingredients	composition
Auramine O	0.1g
Ethyl alcohol, 95%	10ml

Phenol

Ingredients	composition
Phenol crystals	3g
Distilled water	87ml

Decolorising agents and counter stain were same as that of ZN staining techniques.

Appendix-4: Statistical analysis

Statistical tool- chi- square (2) test

1. ZN and Fluorescence Microscopy finding

	Fluorescence Microscopy		
ZN Microscopy	Positive	Negative	Total
Positive	138	2	140
Negative	22	2430	2452
Total	160	2432	2592

Here, the null hypothesis (Ho) is that there is no significant difference between result obtained by ZN microscopy and Fluorescence microscopy.

We have, $^2 = (|ad-bc|-n/2)^2*n/ (a+b) (a+c) (c+d) (b+d) = 2164.69$

d.f., v = (r-1) (c-1) = (2-1) (2-1) = 1

Tabulated value of ^{2} at 5% level of significance for 1 degree of freedom is 3.84.

Decision: since the calculated value of ^{2} is greater than its tabulated value, null hypothesis is rejected i.e. the difference between result obtained is significant.

2. Sputum smear finding by ZN and Fluorescence microscopy

	Fluorescence Microscopy		
ZN Microscopy	Positive	Negative	Total
Positive	131	2	133
Negative	22	2337	2359
Total	153	2339	2492

Here, the null hypothesis (Ho) is that there is no significant difference between result obtained by ZN microscopy and Fluorescence microscopy.

We have, $^{2} = (|ad\text{-}bc|\text{-}n/2)^{2}*n/\ (a+b)\ (a+c)\ (c+d)\ (b+d) = 2062.71$

d.f., $v = (r\text{-}1)\ (c\text{-}1) = (2\text{-}1)\ (2\text{-}1) = 1$

Tabulated value of ^{2} at 5% level of significance for 1 degree of freedom is 3.84.

Decision: since the calculated value of ^{2} is greater than its tabulated value, null hypothesis is rejected i.e. the difference between result obtained is significant.

Appendix 5

Comparison of reporting of Fluorescence and ZN microscopy result

ZN acid fast microscopy	Fluorescence acid fast microscopy					
	Neg	3-9	1+	2+	3+	Total
Neg	**2430**	10	12			2452
3-9	2	**3**	6			11
1+			**65**	10	5	80
2+			1	**13**	9	23
3+			1		**25**	26
Total	2432	13	85	23	39	2592

Neg = negative, 3-9= 3-9 AFB per 100 fields, 1+ = 10-99 AFB per 100 fields, 2+ = 1-10 AFB per field, 3+ = >10 AFB per field.

Both techniques were in agreement that 2430 (93.75%) were negative smears. Higher grades of positive smears were noted by the FM technique. Only two smears were AFB positive by Z-N with a negative FM result. Both stains gave identical results in 2536 (2430+106) of the 2592 pairs of smears (97.84 % agreement).

Appendix 6

AFB Positive Results of ZN and Fluorescence Microscopy

AFB Positive in Sputum Sample							
S.N	Sex/Age	ZN Microscopy			Fluorescence Microscopy		
		1	2	3	1	2	3
1.	M/75	3+	3+	3+	3+	3+	3+
2.	M/30	3+	2+	3+	3+	2+	3+
3.	M/60	3+	3+	3+	3+	3+	3+
4.	M/24	3+	3+	3+	3+	3+	3+
5.	F/58	3+	3+	3+	3+	3+	3+
6.	M/30	N			1+		
7.	M/42	2+	2+	2+	2+	2+	2+
8.	M/18	1+	1+	1+	1+	1+	1+
9.	F/30	3+	3+	3+	3+	3+	3+
10.	M/29	1+	2+	2+	2+	2+	2+
11.	M/60	2+			2+		
12.	M/65	1+			1+		
13.	F/22	N	N	1+	N	7 bacilli	1+
14.	F/60	1+	1+	1+	1+	1+	1+
15.	F/53	1+	N	1+	1+	8 bacilli	1+
16.	F/39	1+	1+		1+	1+	
17.	M/45	1+	1+	1+	1+	1+	1+
18.	M/48	2+	1+	2+	3+	3+	3+
19.	M/75	3+	3+	3+	3+	3+	3+
20.	F/20	6 bacilli	1+	3+	1+	3+	3+
21.	F/45	N	1+	7 bacilli	N	1+	1+
22.	M/20	1+	1+	N	1+	1+	1+
23.	M/18*	N			6 bacilli		
24.	M/25	1+	N		1+	N	
25.	M/30	1+	1+	N	2+	1+	1+
26.	F/16	1+			1+		
27.	M/70	N	N	1+	N	6 bacilli	1+
28.	F/30	N	1+	1+	1+	1+	1+
29.	M/44	N	N		N	4 bacilli	
30.	M/50	1+	7 bacilli	1+	1+	1+	1+
31.	F/29	N	4 bacilli	N	N	N	N
32.	M/25	N	1+	1+	1+	1+	1+
33.	M/14	1+	1+	2+	2+	3+	3+
34.	M/25	1+	2+	1+	1+	1+	2+
35.	M/43	2+	2+	2+	3+	3+	2+
36.	M/21	1+	1+	N	1+	1+	N

37.	M/41*	3 bacilli	1+		8 bacilli	1+	
38.	F/50	2+	2+	2+	2+	2+	2+
39.	M/26	1+	1+	1+	1+	1+	1+
40.	M/50	N	3+	1+	1+	1+	1+
41.	M/25	1+	1+	N	1+	2+	1+
42.	F/50	N	N	1+	1+	8 bacilli	3+
43.	M/30	N	1+		N	1+	
44.	M/21	1+	1+		1+	1+	
45.	F/40*	N			1+		
46.	F/28	1+	2+	2+	1+	2+	3+
47.	M/17	N	1+	N	1+	1+	5 bacilli
48.	M/78	N	6 bacilli	1+	N	1+	1+
49.	M/25	1+	1+	1+	2+	2+	2+
50.	M/54	N	1+	1+	8 bacilli	1+	2+
51.	M/15	1+	1+	1+	2+	1+	1+
52.	M/35	6 bacilli	1+	1+	8 bacilli	1+	3+
53.	M/56	2+	2+	3+	2+	3+	3+
54.	M/55	1+	N	1+	1+	6 bacilli	1+
55.	M/27	6 bacilli	8 bacilli	1+	1+	1+	1+
56.	M/81	6 bacilli	N		6 bacilli	5 bacilli	
57.	M/60*	N			1+		
58.	F/15	1+	1+	1+	1+	1+	1+
59.	F/19	4 bacilli	N	N	N	N	N
60.	F/30	N	1+	1+	N	1+	1+
61.	M/24	3+	2+	3+	3+	3+	3+
62.	M/46	1+	3+	1+	1+	3+	3+
63.	F/15	1+	1+	N	1+	1+	1+
AFB Positive in Extra Pulmonary Sample (Urine Sample)							
64.	M/38	1+	1+	1+	1+	1+	1+
65.	F/35	N	1+	1+	N	1+	1+
66.	F/31	1+			1+		
AFB Positive in Extra Pulmonary Sample (Elbow Aspirate)							
67.	M/25	1+			2+		

*= Follow up Patients

Patients providing sputum samples

	Three samples	Two samples	One sample	Total
Number of patients	748	71	106	925
Number of samples	2,244	142	106	2492

Patients providing extra pulmonary samples

	Three samples	Two samples	One sample	Total
Number of patients	3*	0	91	94
Number of samples	9	0	91	100

*= all 3 patients providing 9 specimens (3 from each) from urine sample