# CHAPTER-I

## 1. INTRODUCTION

Tuberculosis is chronic bacterial infection caused by *Mycobacterium tuberculosis* and characterized by the formation of granuloma in infected tissue as a result of cell mediated hypersensitivity (Thomas, 1994).

Tuberculosis is occasionally caused by *Mycobacterium bovis* and *Mycobacterium africanum*. These organisms are also known as tubercle bacilli (because they cause lesions called tubercles) or as acid –fast bacilli (AFB). The bacilli resist decolourization by dilute mineral acids and alcohol (Harries et al., 2004).

Tuberculosis is humanity's greatest killer which is out of control in many parts of the world. The disease is preventable and treatable but it has been grossly neglected and no country is immune to it. The diagnosis of TB infection is vital both clinically and epidemiologically (Barez et al., 1995). Early diagnosis, effective treatment, and successful cessation of transmission are major strategies in the control of TB (NTC, 2002/2003).

Tuberculosis is the major health problem in the SAARC region causing an immense burden of disease (Kumar and Bam, 1996). About 60% adult population has been infected with tuberculosis bacilli in Nepal. Every year 2, 00,000 people develop tuberculosis and 13,000-15,000 people infected with tuberculosis die. One infected patient can transmit the disease to 10-15 people every year and at least 5% of newly diagnosed patient with TB have resistance to one or more drugs (Sharma and Smith, 1996).

The latest survey conducted in 2001/2002 showed MDRTB of 1.3%. In 2002, 2.4% of TB patients were infected with HIV (NTC, 2007). The SAARC region accounts more than 29% of global burden of TB with 0.6 million deaths every year and 2 million new

cases annually (Bam et al., 2002). Introduction of treatment by DOTS has already reduced the death number; it is estimated 5,000-7,000 people still die per year from TB (NTC, 2007). TB control is high on the international public health agenda, not just because of enormous burden of diseases, but also because of short course chemotherapy is recognized to be among the most cost effective of all health interventions (Dye et al., 2010).

Transmission occurs by air borne spread of infectious droplets. The source of infection is a person with pulmonary TB who is coughing, with usually sputum smear positive (Harries et al., 1998).

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

Tuberculosis diagnosis is performed clinically, radiologically and bacteriologically (Deun, 2001). The diagnosis may be strongly suspected clinically with typical cavitary or calcified lesions are detected radiographically, but confirmation requires isolation of bacilli from the lesion (Chakraborty, 2005). The core stone of laboratory diagnosis of TB is the direct microscopic examination for tubercle bacilli (WHO, 1998 a). The history of sputum examination dates back to march 24, 1882 when Robert Koch discovered the tubercle bacillus and confirmed the bacterial etiology of Tuberculosis and also presented methods for both staining and culturing (Ponticeellio et al., 2001).

Sputum smear positive patients are the most potent sources of transmission of *Mycobacterium tuberculosis* in the community. Sputum smear positive patients are much

likely to transmit *Mycobacterium tuberculosis* than patients negative on sputum smear examination. Thus, sputum smear microscopy is one of the most effective and efficient tools for case finding in a National TB Control Programme. It identifies the cases that should have the highest priority in TB control. Moreover, sputum smear microscopy is highly specific in identifying acid-fast bacilli, which in high prevalence countries are almost always diagnostic of tuberculosis. It is the basic diagnostic tool in tuberculosis control.

The bacilli in the sputum can be detected microscopically by 3 types of staining procedures for rapid detection and confirmation of AFB. Fluorochrome, Ziehl-Neelsen and kinyoun (Kar et al., 2003; Balows et al., 1991). Fluorescence microscopy is on average 10% more sensitive than bright field microscopy in detecting TB in sputum smears. This study concentrates on TB screening using bright-field microscopy of ZN-stained sputum smears, as this is the method of choice in developing countries, due to the low cost and ease of equipment maintenance compared to fluorescence microscopy; low cost fluorescence microscopes have however become available (Khutlang et al., 2010).

Ziehl-Neelsen, a carbol fuchsin based stain (hot process) is a standard staining technique for staining of sputum smears for detection of *Mycobacterium tuberculosis* (Pandey et al., 2009). Smears stained by ZN method can detect bacilli when they are at the order of $10^5$/ ml of sputum (Forebs et al., 1998; Katoch, 2004). Revised National Tuberculosis Control Programme (RNTCP) guideline recommended the use of 1% carbol fuchsin in ZN method. However recent World Health Organization (WHO) guidelines recommended using carbol fuchsin at a concentration of 0.3%. However, the reasons for reducing concentration of carbol fuchsin from 1 to 0.3% are not documented. The efficacy of 0.3% carbol fuchsin over 1% carbol fuchsin in ZN staining method has not been studied previously (Selvakumar et al., 2002).

The present study about the comparative evaluation of use of different concentrations of carbol fuchsin used in ZN staining procedures will help to distinguish appropriate

concentration of carbol fuchsin to be used for demonstration of AFB in laboratory and public set-up on the basis of sensitivity and specificity obtained in each staining techniques with the reference to sputum culture.

# CHAPTER – II

## 2. OBJECTIVES OF THE STUDY

### 2.1 GENERAL OBJECTIVE

To evaluate ZN-staining techniques using different concentrations of carbol fuchsin for the detection of Acid Fast Bacilli (AFB) in direct sputum smears.

### 2.2 SPECIFIC OBJECTIVES

a. To perform ZN staining technique using 0.3% carbol fuchsin.
b. To perform ZN staining technique using 0.5% carbol fuchsin.
c. To perform ZN staining technique using 1% carbol fuchsin.
d. To perform culture and biochemical identification of the isolates.
e. To determine the sensitivity and specificity of each ZN staining techniques with reference to the culture.

# CHAPTER - III

## 3. LITERATURE REVIEW

### 3.1 Tuberculosis

Tuberculosis is a major public health problem in many developing countries and the world Health Organization (WHO) declared it a global emergency (Selvakumar et al., 2004). The disease primarily affects lungs and causes pulmonary tuberculosis (PTB). It can also affect intestine, meninges, bones and joints, lymph glands, skin and other tissues of the body (American Thoracic Society, 1990).

Most infections in humans result in an asymptomatic, latent infection and about one in ten latent infections eventually progress to active diseases, which if left untreated, kills more than 50% of the victims.

Tuberculosis is a socio-medical problem. The SAARC region accounts more than 29% of global burden of tuberculosis with 0.6 million deaths every year and 2 million new cases annually (Subba et al., 2009).

According to the latest World Health Organization (WHO) report in 2005, there were 8.8 million new TB cases and 1.6 million deaths were attributed to the diseases worldwide. The situation becomes more complicated due to the rising human immunodeficiency virus/AIDS pandemic, the emergence of multidrug-resistant TB and the recently described extensively drug resistant TB (Affolabi et al., 2008).

Multi drug resistant (MDR) tuberculosis is caused by *Mycobacterium tuberculosis* that is resistant at least to Isoniazid and rifampicin, and XDR tuberculosis by *Mycobacteria* resistant to rifampicin and isoniazid, any fluoroquinolone, and one of the three injectable drugs, capreomycin, kanamycin and amikacin. In 2008, 5.7 million (61%) of the estimated 9.4 million new and relapsed tuberculosis cases were identified and treated on the basis of the WHO stop TB strategy. Drug resistance severely threatens tuberculosis control, since it raises the possibility of a return to an era in which drugs are no longer effective. Tuberculosis control efforts are complicated by weak programmes with poor access to laboratory diagnosis and effective treatment. Investment in laboratory capacity and staff and the introduction of new rapid diagnostic tests are crucial (Gandhi et al., 2010).

### 3.2 Global history

Tuberculosis has been present in human since antiquity. The earliest unambiguous detection of *Mycobacterium tuberculosis* is in the remains of bison dated 18,000 years before the present. Whether tuberculosis originated in cattle and transferred to humans, or diverged from a common ancestor infecting a different species, is currently unclear. Tubercular decay has been found in the spines of Egyptian mummies. Evidences of tuberculosis lesions of bone have also been found in Egyptian mummies dating back to 3400 BC (Keers, 1978).

J.L.Schonlein is credited to have named the diseases “tuberculosis” (Rossenblatt, 1973). The word “tuberculosis” means “a small lamp” (Dubos and Dubos, 1952). In the past, tuberculosis has been called consumption, because it seemed to consume people from within, with a bloody cough, fever, pallor, and long relentless wasting. Other names included Phthisis (Greek for consumption) and Phthisis Pulmonalis; scrofula (in adults), affecting the lymphatic system and resulting in swollen neck glands; tabes mesenterica, TB of abdomen and lupus vulgaris, TB of the skin; wasting diseases; white plague, because sufferers appear markedly pale; king’s evil, because it was believed that a king’s touch would heal scrofula; and pott’s diseases, or gibbus of the spine and joints. TB is also called Koch’s diseases after the scientist Robert Koch. Tuberculosis has also been referred to as the “great whit scourge” and by john Bunyan as “the captain of all these men of death”. In ancient Hindu texts, tuberculosis is referred to as Rograj and Rajayakshma meaning wasting diseases (Grange, 1990).

Exact pathological and anatomical descriptions of the diseases began to appear in the seventeenth century. In this opera Medica of 1679, Franciscus sylvius, the Duth physician was the first to identify actual tubercles as a consistent and characteristic change in the lungs and other areas of consumptive patients. He also described this progressing to abscesses and cavities. Hence Sylvius deduced from autopsies that tuberculosis was characterized by the formation of nodules, which he termed “tubercles” (Lowell, 1966).

The modern knowledge of tuberculosis started from the work of Rene Theodore Laennec (1781-1826) a French clinician, who himself was a consumptive and succumbed to the diseases. In 1819, he invented the stethoscope which helped in accurate description of tuberculosis lesions; he described follicular (miliary) and infiltrative (exudative) forms of tuberculosis (Rao et al., 1981). Transmissible nature of tuberculosis was established by

Jean-Antoine Villemin (1827-1892), a French military Surgeon. The staining technique was discovered by Robert Koch in 1882, March 24, which enabled him to see *Mycobacterium tuberculosis*, and thereby announced the discovery of tubercle bacillus and succeeded in culturing it in inspissated serum. The acid fast nature of the organism was discovered by Ehrlich in 1882 and the present method of acid fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence the name Ziehl-Neelsen stain (Lowell, 1966).

X-rays, which was discovered in 1795 by the professor Roentgen, were put to clinical use by 1904. The findings of radiology and bacteriology helped in developing further knowledge of the disease and correlation between them (Rao et al., 1981).

Though the disease has been identified earlier, the modern era of tuberculosis treatment begun only in 1946 with the advent of Streptomycin and in 1952 with development of Isoniazid hydrochloride (INH). Since, then the modalities of treatment regimens were constantly revised and updated. Despite the availability of effective chemotherapy, it is still a major public health problem in the most countries of the world (Stewart and Beswick, 1977).

## 3.3 Types of Tuberculosis

### 3.3.1 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 (Fitzgerald et al., 2005).

**Primary infection:-**

Primary Tuberculosis is the initial infection by Tubercle bacilli in a host. The bacilli which are engulfed by alveolar macrophages multiply and give rise to a sub pleural focus of tuberculosis Pneumonia, commonly located in the upper part of the lower lobe or lower part of the upper lobe. The involved area is called the Ghon focus. The Ghon focus together with enlarged hilar lymph node constitutes ‘the primary complex’. This occurs 3-8 weeks from the time of infection and is associated with the development of tuberculin hypersensitivity. A few bacilli may survive in the healed lesions and remains latent (Fitzgerald et al., 2005).

**Post primary tuberculosis:-**

It is also called secondary or adult type of Tuberculosis which is due to reactivation of latent infection. It affects mainly the upper lobes of the lungs and the lesions undergo necrosis and tissue destruction, leading to cavitation. In the immunodeficient individuals instead of cavity formation there is widespread dissemination of lesions in the lungs and other organs (Fitzergald et al., 2005).

**Symptoms of Pulmonary Tuberculosis:**

Tuberculosis may present without symptoms, for example, when an individual who has been in contact with an infectious case is screened and found to have disease. Up to 50% of childhood tuberculosis may be detected in this way. Cough, malaise, fever, weight loss, night sweats, breathlessness and chest pain are characteristic symptoms of Pulmonary Tuberculosis in approximate decreasing order of frequency (Fitzergald et al., 2005).

**3.3.2 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, 2003). Common forms of extra pulmonary TB include the following: lymphadenopathy, Pleural effusion TB, meningitis, miliary, intestinal, bone, urogenital, skin and eye TB (NTC, 1997). Patients usually present with constitutional features (fever, night sweats, weight loss) and local features related to the site of diseases.

**3.4 Classification**

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

1) Strict Pathogens

*M. tuberculosis* complex

| | | |
|---|---|---|
| *M. tuberculosis* | ⟶ | human type |
| *M. bovis* | ⟶ | bovine type |
| *M. africanum* | ⟶ | human type |

*M. microti* → Murine type

2*) Leprae bacilli*

*M. leprae* → causing leprosy in man

*M. leprae murinum* → causing rat leprosy

3) Other animal pathogens

*M. microti* → Murine type

*M. paratuberculosis* → Johne's bacillus

4) Atypical *Mycobacteria*

Runyon group I photochromogens

Runyon group II Scotochromogens

Runyon group III Non-photochromogens

Runyon group IV Rapid growers

5) Saprophytic *Mycobacteria* (non-pathogenic)

*M. phlei* → present in grass

*M. stercoris* → present in dung

## 3.5 Bacteriology

### 3.5.1 Etiological agent

The etiological agent of tuberculosis is *Mycobacterium tuberculosis.* The genus *Mycobacterium* belongs in the Mycobacteriaceae family, Actinomycetales order and Actinomycetes class. *Mycobacterium* are very thin rod shaped (0.2 to 0.4×2 to 10µm), and non-motile. Genera that are closely related to members of the genus *Mycobacterium* include *Nocardia, Rhodococcus and Corynebacterium*. *Mycobacterium spp*. has an unusual cell wall structure that contains N-glycolylmuramic acid in lieu of N-acetylmuramic acid and has very high lipid content. Because of this cell wall structure, *Mycobacteria* are difficult to stain with commonly used basic aniline dyes, such as those used in gram stain. However, these organisms resist decolourization by acidified alcohol (3% hydrochloric acid). This important property of *Mycobacteria* that is dependent on its cell wall is referred to as acid-fastness. Another important feature of this organism is that they grow more slowly than most other human pathogenic bacteria because of their hydrophobic cell surface. Because of this hydrophobicity, organisms tend to clump, so that

nutrients are not easily allowed into the cell. Growth is slow or very slow, with colonies becoming visible in 2 to 60 days at optimum temperature (Forebs et al., 2007).

*Mycobacterium tuberculosis* is an obligate aerobe, grows optimally at 37°C (range 25°C to 40°C) and pH 6.4 to 7. It is a slow organism with generation time of 14 to 15 hours (Forbes et al., 2007). *Mycobacterium* are more resistant to drying and chemical disinfectant, temperature 60°C for 20 minute can kill it, moist heat at 100°C kill it readily. When exposed to sunlight the culture may be killed in 2 hours. In sputum it survives 20 to 30 hours even in sunlight. Phenol solution 5% kills in 24 hours (Gupta and Prakash, 2002).

The virulence of the tubercle bacilli is due to its resistance to cells and fluids rather than to the production of toxic substance (Grange, 1990). The important characteristics feature of the tubercle bacilli for its virulence includes slow growth dormancy, complex cell envelope, intracellular pathogenesis and genetic homogenecity. Novel biosynthesis pathways generate cell wall components such as mycolic acid, mycocerosic acid, phenolthiocerol, lipoarabinomannan, arabinogalactan and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis (Collee et al., 1996).

**3.5.2 Pathogenesis:**

A microbes become a pathogen when its biochemical pathways either individually or acting in concert with one another, causes diseases in a host. In microbial pathogenecity, two terms encountered are infection and diseases. Infection refers to the multiplication (or colonization) or the persistence of the organism within the host environment, while disease refers to the significant damage caused by the organism in the host due to the infection. Microbial pathogenecity is usually not attributed to a single contributing factor. It is multifactorial (Manjula and Sritharan, 2002).

Source of infection is an open case of pulmonary tuberculosis whose sputum may contain more than 10,000 bacilli per ml. Most of the inhaled bacilli are arrested by natural defenses of the upper respiratory tract. On reaching the lungs, the bacilli are ingested by alveolar macrophages. The virulence of tuberculosis is related to their ability to survive and multiply in macrophages. Only about 10% of those infected develops active tuberculosis (Manjula et al, 2002; Smith, 2003). There is production of characteristic

lesions called ‘tubercle’ in the infected tissue. These are avascular granulomas composed of a central zone containing giant cells, with or without caseation, and a peripheral zone of lymphocytes and fibroblasts (Fitzergald et al., 2005).

Cell mediated immunity is the important immune mechanism. Humoral immunity does not have much role. Since the main route of entry is respiratory route, the alveolar macrophages are the important cell type that combats the pathogen. It forms the main component of innate immune response. The various aspect of macrophage-mycobacterium interaction include the binding of *M. tuberculosis* to macrophage via surface receptors, phagosome-lysosome fusion, Mycobacterial growth inhibition or killing through free radical base mechanism (such as reactive oxygen and nitrogen intermediates) and cytokine-mediated mechanisms macrophages are also involved in recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T-cells for development of acquired immunity. Other components of the innate immune response include natural resistance associated macrophage protein (NRAMP), neutrophils and natural killer cells (Raja, 2004).

Predisposing factor for progression of disease are old age, alcoholism, diabetes, neoplastic diseases, malnutrition, immunosuppressive drugs, stress and drug induced congenital or acquired immunodeficiency including HIV infection (Groothius and Yates, 1991).

### 3.5.3 Transmission

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

Transmission generally occurs indoors where droplet nuclei can stay in the air for long time (WHO, 1996). It is well established that direct sputum smear positive patients are the principal source of infection (Roullion et al., 1976). The threshold for detecting bacilli on light microscopy is about $10^4$ bacilli/ml while the infecting dose of *M. tuberculosis* is estimated to be fewer than 10 orgranisms and report does show that patient with smear negative culture positive TB appears to be responsible for about 17% of TB transmission (Behr et al., 1999).

Tuberculosis is not transmitted by fomites such as dishes and other articles used by patients. Sterilization of these articles is of little or no value. Patient with extra pulmonary tuberculosis or smear negative tuberculosis constitute a minimal hazard for transmission (Park, 2002).

### 3.6 Diagnosis

The timely identification of person infected with *M. tuberculosis* and their rapid laboratory conformation of tuberculosis are two key ingredients of effective health measure for control of the TB (Noordeen and Godul, 1988). Ideal TB diagnosis should address the following four focal aspects of TB control in low-income countries.

1) It should improve case detection for both smear positive and smear negative cases, simplify, speed up detection of drug resistance and also detect cases of preclinical diseases or latent infection.

2) It should be patient friendly so that it requires minimum number of patient visits to the clinics.

3) It should be simple such that it can be administered by general health service technician with minimum skills and require minimum supervision.

4) It should be based on consumables that are stable at room temperature and require minimum technical infrastructure. The consumables for the diagnosis should be available in a country wide basis and on a long-term (Kar et al., 2003).

#### 3.6.1 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, fatigue, night sweat (during sleep) and fever (WHO, 1997). The diseases in the children are 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. The symptoms of extra pulmonary tuberculosis depend on the organ involved. Clinical sign and symptoms develop in only a small proportion (5-10%) of infected healthy people (Enarson et al., 1996).

#### 3.6.2 Radiological diagnosis

Chest x-rays (radiology) can also help in the detection of pulmonary tuberculosis but they do not allow etiological diagnosis. TB is difficult to diagnose with certainty on X-ray

alone. X-rays are expensive, unreliable as patients are often treated for TB when they do not have it. But X-ray is sometimes needed for difficult individual problems in particular for HIV infection (Issaelbacher et al., 1992). There is radiological difference between primary and secondary tuberculosis. Primary diseases are usually characterized by a single lesion in the middle or lower right lobe with enlargement of the draining lymph nodes (McMurray, 2001).

### 3.6.3 Tuberculin skin test

The intracutaneous tuberculin skin test is indirect test method for diagnosis of tuberculosis and reliable means of recognizing prior *Mycobacterium* infection. It is useful for identifying persons infected with *M. tuberculosis* complex (MTBC), but does not differentiate active diseases from infection. Intradermal introduction of PPD (purified protein derivative) into a previously infected hypersensitive person results in the delayed (48-72 hours) appearance of an indurated (raised, hard) reaction with or without erythema (McMurray, 2001). Despite these limitations, the tuberculin skin test is still very useful tool, especially when conversion to a positive skin test is used to identify recently infected persons for preventive therapy or to help confirm a physician's suspicion of tuberculosis (Shinnick and Jonas, 1994).

### 3.6.4 Laboratory diagnosis

**a. Specimen collection and transport**:

Specimen consist of fresh sputum, gastric washings, urine, pleural fluid, cerebrospinal fluid, joint fluid, biopsy material, blood or other suspected material. Sputum specimens must be free of food particles, residues and other extraneous matter (Forebs et al., 2007).

About 85% of cases are pulmonary and most usual specimens for diagnosis of Pulmonary tuberculosis is sputum (Baas et al., 1990). A good specimen of sputum should be mucopurulent, thick, yellow and sticky. It is the first choice in investigation of Pulmonary disease and should be collected whenever possible, preferably before commencement of chemotherapy (Collins et al., 1997). The examination of three specimen increases the predictive value of positivity of smear microscopy, reaching almost to that of culture

(Chonde et al., 2000). The overnight specimen is more likely to be positive than the spot specimens (NTP, 2002).

Specimens should be sent promptly to the laboratory to avoid being overgrown with organisms other than *Mycobacterium.* If delay is unavoidable, specimens sent should be stored in the refrigerator. Specimens sent through the post in warm weather should be packed in dry ice or with an ice pack (Collee et al., 1996). Specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing. The cetylpyridinium (CPC) method is widely used for the transport of sputum specimens (Smithwick, 1995). Sodium carbonate was also found to be a better preservative of sputum specimens for AFB smear microscopy as well as culture (Bobadilla, 2003).

**b. Sputum Smear Microscopy**

Although much work is being done to develop new diagnostics, in most resource-limited countries direct sputum smear microscopy remains the primary means for diagnosis of TB. Given the known limitation of smear microscopy, considerable effort has been given to identifying methods that can optimize the yield and accuracy of smear microscopy. Mobile phone-based microscopy and automated detection systems using image processing are other novel approaches that have been proposed although the use of these approaches is yet to be adequately validated (Pai et al., 2009).

Sputum Smear Microscopy is the mainstay of diagnosis of TB. It is efficient and can confirm the disease. Besides this, other advantage of AFB microscopy are well known. It is inexpensive to perform and is very specific in high prevalence settings. For these reasons, microscopy rightly retains the primary role in case detection as it can detect the most infectious subset of patients (Selvakumar, 2003; Parekh and Kar, 2003).

The detection of acid- fast bacilli (AFB) in stained smear examined microscopically is the first bacteriologic evidence of the presence of *Mycobacterium* in a clinical specimen. It is the fastest, easiest, quickest and least expensive tool for the rapid identification of potentially infectious TB patients (McMurray, 2001) and it provides the physician with a preliminary conformation of the diagnosis. The main value of AFB microscopy lies in its speed and extremely high specificity, while the main disadvantage is said to be its low

sensitivity. Sensitivity of microscopy is increased from 51% to almost 100% by introducing the cytocentrifugation (Kox, 1996).

The sensitivity of a single sputum AFB smear is 30% to 40% because direct microscopy cannot distinguish between *M. tuberculosis* and non-tuberculosis mycobacterium (NTM), specificity is a concern but studies have shown that the AFB smear continues to have a high specificity (90%). The sensitivity of AFB smear is such that it requires 6000 to 10,000 organisms per ml of sample to register as positive. While this limits the sensitivity of microscopy, it also serves to identify patients who are highly infectious and therefore the highest priority for immediate anti-TB treatment (Lam et al., 2010).

Gebre et al., 1995 reported that the sensitivity of sputum microscopy can be significantly augmented after liquefaction of sputum with sodium hypochlorite (NaOCL), commonly known as household bleach followed by centrifugation. As a potent disinfectant NaOCL also kill *Mycobacterium* and thus eliminates the risk of laboratory infection, a risk that cannot be neglected, especially in laboratories with inadequate safety standards.

Although 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 prevalaence and severity of diseases, the type of specimen, the quality of specimen, collection, the number of *Mycobacterium* present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, the staining technique and the quality of examination (Somoskovi et al., 2001).

Whenever diseases are suspected, three specimens must be collected for examination by microscopy. Whenever possible they should be obtained within twenty-four hours. The overnight specimen is more likely to be positive than the spot specimens. The cumulative positivity is 31%, 93% and 100% for first, second and third sputum respectively (NTP, 2002).

Respiratory specimen 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 must be detected by acid-fast smear examination (Kar et al., 2003; Balows et al., 1991).

When all culture positive specimens are considered, the sensitivity of the direct smear compared to that of a smear made from the concentrated specimen was significantly

different (Peterson et al., 1999). The sensitivity against culture was significantly higher with the concentration method (80%) than with the classic direct smear method (57%) (Garay, 2000). However this concentration method involves incubation at constant temperature diluting with distilled water and high speed centrifugation. These conditions are not often met by diagnostic facilities in rural areas of developing countries.

When acid-fast organisms are observed on a smear, results must be quantified to be meaningful because this quantization 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).

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, *Mycobacterium* 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%.

However, the reliability of the direct sputum smear examination as a single test for the diagnosis of TB has earlier been questioned. It is estimated that 60% to 70% of all tuberculosis cases is diagnosed through sputum smear examination. The low yield by smear may be attributed partly, to the few numbers of bacilli present in a sputum specimen which greatly increases the chance of obtaining a false negative. Smear positivity has been found to be affected by the number of bacilli in a sputum specimen (Githui et al., 1993).

For developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods like demonstration of acid-fast bacilli in smears is great importance. No other diagnostic tool offers the affordable as well

as efficiency in diagnosis of tuberculosis in public health setup, as sputum microscopy does. 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 (Shrestha et al., 2005).

**i. Fluorescence Microscopy**

Fluorescence microscopy was introduced by Hageman (1937), who originally used berberine sulphate as the dye but later (1938) recommended auramine. Though fluorescence microscopy is credited with increased sensitivity, there is concern that specificity may be lower as there is possibility of false positive results because inorganic objects may incorporate fluorochrome dyes (Traunt et al., 1962, Steingart et al., 2006 b). So, for the widespread implementation of fluorescence microscopy in tuberculosis endemic countries along with its sensitivity, its specificity should be determined with its modifications in the technique. Current fluorescence microscopy technique needs to be optimized to generate higher than usual sensitivity and yields (McCarter and Robinson, 1994).

McCarter and Robinson (1994) concluded fluorescence staining at 37°C increases overall smear sensitivity and enable the visualization of greater numbers of AFB in a smear. 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 result of the smear microscopy should be reported according to an internationally agreed quantization scale (Waard, 2007).

From an operational perspective, this is highly advantageous, particularly when high numbers of samples are screened per day because the majority of laboratory time is spent confirming negative smear results (Marais et al., 2008).

**ii. ZN Microscopy**

ZN method has been commonly used particularly in developing countries, because of its simplicity and low cost (Githui et al., 1993). It is also called “hot stain” and its modification, kinyoun method is also called “cold stain”(Cheesbrough, 2000).

The “hot stain” requires application of heat to the fixed smears flushed with the stain during staining process The Kinyoun stain is a modification of classical ZN method that

excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin and phenol or the addition of detergent (Somoskovi et al., 2001). It is a two step cold staining method which requires concentrated carbol fuchsin as a primary stain and Gabbet methylene blue as a counter stain (Bhatt and Bhatt, 2000). In carbol fuchsin (Ziehl-Neelsen) procedure, acid fast organism appears red against blue background. It is the modification of Ehrlich's (1882) original method. In ZN stained smears, AFB typically appears as purple to red slightly curved rods (1-10 µm×0.2-0.6µm) that occasionally are beaded or banded but also may appear coccoid or filamentous.

This is the most popular method of acid fast staining. The primary stain used is concentrated carbol fuchsin. Heat along with phenol present in carbol fuchsin helps in the penetration of the dye. 25% sulphuric acid acts as the decolouriser. It is counterstained by 0.1% methylene blue. The bacilli are stained pink and the background along with pus cells and other bacteria appear blue. Ziehl-Neelsen method of staining has a sensitivity and specificity of 33.79% and 100% respectively (Watt et al., 1996).

Various studies have been done using different concentrations of carbol fuchsin to find out the appropriate concentration of carbol fuchsin in detecting AFB. In the study conducted by Selvakumar et al., 2002, the use of 0.3% carbol fuchsin recommended by WHO was found inferior to 1% carbol fuchsin recommended by RNTCP. The studied showed that use of 0.3% carbol fuchsin may result in 20% of smear positive patients being missed. The use of 0.3% carbol fuchsin is less sensitive and no more specific in detecting AFB in sputum. The modified ZN method was found to be significantly less sensitive than the standard ZN method that uses 1% carbol fuchsin. The modified ZN method has 11% more smear negative culture positive samples than the standard ZN method.

In a study done by Selvakumar et al., 2005, duplicate smears from 416 samples were stained with ZN method set with 1% basic fuchsin and the other 0.3%. Another set of duplicate smears from 398 samples were stained with ZN, one with 1% basic fuchsin and the other 0.1%. The coded smears were read and discrepancies resolved. All samples underwent Mycobacterial culture. The sensitivity of ZN using 0.3% (65%), and 1% basic fuchsin (62%) was comparable, while it was reduced using 0.1% (74%) compared to 1% basic fuchsin (83%). Reducing the concentration of basic fuchsin below 0.3% in ZN staining was found to significantly reduce its sensitivity.

In another study by Deun et al., 2005 sensitivity with ZN 1% 5 minute staining was not significantly higher than with 0.3% 5 minute staining (89.9% Vs 86.5%). Routine examination using 1% carbol fuchsin for 15 minute identified more positive than any of the study techniques. Furthermore, in kinyoun method using stain with high (3%) basic fuchsin and phenol concentration (6.25%) made staining for only 5 minute without heating possible.

Carbol fuchsin an important component of Ziehl-Neelsen stain, is a mixture of phenol and basic fuchsin. It is also used as a topical antiseptic. It is also known as castellan's paint in the U.S. The commercially available "basic fuchsin" is either the chloride or acetate of pure pararosaniline or consists of variable mixtures of it with higher homologues. Consequently, only a basic fuchsin which has an absorption maximum at $\lambda \geq 552$ nm could be employed for the acid-fast stain of *Mycobacterium* in a stable manner. Acid-fast staining of *Mycobacterium* in the form of beadings is obtained by means of a carbol fuchsin solution (Ziehl-Neelsen stain) prepared from pararosaniline or from certain kinds of basic fuchsin (Harada et al., 1976).

Basic fuchsin is a mixture of four dyes in different proportions. There are substantial differences between manufacturers and batches with respect to composition, quality and solubility. The original Ziehl-Neelsen (ZN) method used a concentration of 1% basic fuchsin in the carbol fuchsin. It is historically not clear what lead to current reduced concentration of 0.3% recommended by the WHO, the union and other organization. More operational research is required to compare a 0.3% versus 1% carbol fuchsin concentration (Gilpin et al., 2007).

ZN method is commonly used throughout the world and still remains the standard method against which new tests must be measured. All the techniques are based on the relatively unique property of *Mycobacterium* to retain the primary dye even after exposure to strong mineral acid or acid-alcohol, hence the term, acid-fast bacilli. The smears stained by ZN method can detect bacilli when they are at the order of $10^5$/ml of sputum.

Despite the clear operational advantages of fluorescence microscopy, conventional light microscopy remains the most widely used diagnostic test in resource-limited settings. Various disadvantages of fluorescence microscopy that makes conventional light

microscopy most widely used is its limited lifespan (typically 200-300 hrs) and the high cost of short-arc mercury vapour lamp (MVP), which has traditionally been used as the excitatory light source. Repeated on- and –off switching as may occur with unreliable local power supply shortens the lifespan, even further. In addition, MVP's are the energy inefficient and requires an extensive power supply; they may also fail catastrophically and release toxic mercury into the environment. Moreover, it has high maintenance cost too (Marias et al., 2008). Thus, in sputum smear microscopy, ZN is the most commonly used technique because of its simplicity and low cost (Shrestha et al., 2005).

**c. Limitation of sputum smear microscopy**

Although the stained smear provides rapid results and is economical and simple to perform, it has limitation. Probably the most important limitation is its low sensitivity which in various studies has ranged from 22% to 78% compared with culture. The estimated limit of detection of AFB in sputum is $5\times10^3$ to $1\times10^4$ bacilli/ml of specimen which is considerably lower than that of culture (approximately $10^2$ bacilli/ml). The AFB smear does not allow identification of the infecting *Mycobacterium* to the species level. A smear of sputum or other respiratory specimen that stains positively for AFB, therefore, does not provide an unequivocal diagnosis of Pulmonary TB; this requires culture conformation or the use of a specific and nucleic acid amplification test (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). Besides this, smear examination requires sputum collection, smearing, drying, staining and examination delays in reporting may lead to the substantial difficulties in case holding. The need for duplicate or triplicate sputum examination further compounds the problem (Perkins, 2000).

Many variables influence the results of microscopy which includes the availability of saliva instead of sputum, as well as quality and quantity of sputum. A large work load may also influence the sensitivity. False negativity due to fatigue may also contribute to decreased sensitivity (Parekh and Kar, 2003). Apart from this microscopy cannot diagnose

between live and dead AFB so that some patients excreting non-viable bacilli at the end of treatment may be roughly considered as failure-cases (Deun et al., 2001).

In addition to this, the diagnosis of TB by smear is made more difficult than ever before especially among the HIV/TB patients with low concentrations of bacilli in their pretreatment sputum specimens and also may therefore have negative sputum smears. In view of this, the need for quicker and more sensitive methods than those currently used for diagnosis of TB has become imperative (Githui et al., 1993).

**d. Culture**

The sensitivity of culture is excellent ranging from 80% to 93% and specificity is quite high at 98%. Till date, culture is taken as "gold standard" method for diagnosis of tuberculosis, requiring only 10 to 100 microorganisms to detect *M. tuberculosis* (American Thoracic Society, 2000). It also provides material for further identification, genotyping and drug susceptibility testing (Kent and Kubica, 1985). Digestion and decontamination is done before culture. The most commonly used method is petroff's method in which 4% Sodium hydroxide (NaOH) is used are N-acetyl-L-cysteine along with 2% NaOH, 5% oxalic acid, 13% trisodium phosphate and 1% acetylpyridinium chloride along 2% NaOH.

**i. Types of culture media**

Mainly there are two types of culture media

**Solid Media**

Solid media includes egg based media like LJ and Ogawa and agar based media like Middlebrook7H10 and Middlebrook7H11. This media contains malachite green, a dye that suppresses the growth of contaminating bacteria. Of the egg based media, LJ media is most commonly used in clinical laboratories. Agar based medias are transparent and provides a ready means of detecting early growth of microscopic colonies. The distinctive colony characteristics of *M. tuberculosis* are rough, tough and buff colony (Collee et al., 1996; Rattan, 2001).

**Liquid Media**

Middlebrook 7H9 and Dubos Tween albumin broth are commonly used for sub culturing strains of *Mycobacterium* and preparing of inocula for drug susceptibility tests and other in vitro tests (collee et al., 1996; Rattan, 2001). Examples of broth media include

BACTEC 460TB and BACTEC MB9000 methods, the Mycobacterial growth indicator tube or MGIT non-radiometric method and the manual septic-check AFB system (Brodie and Schluner, 2005).

**ii. Biochemical properties**

**Niacin test**

This test is based on detection of presence of nicotinic acid in the culture medium. Nicotinic acid is an intermediate in the biosynthesis of NAD (Nicotin adenine Dinucleotide). In *M. tuberculosis,* this pathway blocks and nicotinic acid excretes in the culture medium. This test differentiates *M. tuberculosis* (99.5%species) from most other *Mycobacterium.* A positive reaction is shown by yellow colour formation when the fluid extract of the growth is mixed with 4% alcoholic aniline and 10% cyanogine bromide solution. INH test strip is also used for detecting niacin and its metabolite in the aqueous extract of organism of medium. For reliable results, the niacin test should be performed only from cultures on LJ that are at least 3 weeks old and show at least 50 colonies (Killburn and Kubiac, 1968).

**Nitrate reduction test**

In nitrate reduction test, the presence of nitrate (product of the nitroreductase enzyme) is detected. M. *tuberculosis* is suspended on a buffer solution containing nitrate, which gives a pink or red color when treated with sulphanilamide and N-napthylethylene-diamine-dihydrochloride. *M. tuberculosis* is one of the strongest reducers of nitrate among the *Mycobacterium*, which allows for this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria. This test differentiates *M. tuberculosis* from *M. bovis* which does not reduce nitrate (WHO, 1998 b).

**Catalase test**

This test is used to differentiate those bacteria that produce the enzyme catalase from non-catalase producing bacteria. Catalase is an intracellular soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The oxygen bubbles in the reaction mixture indicate catalase activity. Catalase is present in most cytochrome containing

aerobic and facultative anaerobic bacteria. In catalase test at 68°C, the growth is incubated in phosphate buffer (pH 7) at 68°C in a water bath for 20 minutes and chilled to room temperature before addition of Tween 80. *M. tuberculosis* produces less than 45mm bubbles at room temperature but is catalase negative at 68°C (Collee et al., 1996).

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

Reduced flavoprotein — Oxidized flavoprotein

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

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

**3.6.5 Other laboratory diagnostic techniques:**

**a. Immunological diagnostic techniques**

**i. Antigen detection**

Mycobacterial antigen is detectable in clinical specimens by one of specific antibodies in agglutination technique and Enzyme Linked Immunosorbent Assay (ELISA). Development of such test have been overshadowed by DNA technology but results of the few studies of their use with 'clean' specimens such as cerebrospinal, pleural and peritoneal fluids were increasing with high sensitivities and specificities (Wadee et al., 1990).

**ii. Antibody detection**

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

**iii. Elispot test**

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

**b. Molecular Methods**

**i. Polymerase Chain Reaction (PCR)**

PCR, a well developed technique, is used extensively for the diagnosis of TB. It is a DNA based technology which produces relatively large number of copies of DNA molecules from minute quantities of source DNA from viable or nonviable cell material even when the source DNA is of relatively poor quality. PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension. PCR enables the amplification of specific sequences of target nucleic acids. It is not only simple and fast, but also very sensitive and specific to amplify even a single molecule of DNA (Forbes and Hicks, 1993).

With the increased incidence of TB and the advent of MDR-TB strains, the demand of PCR is high in developing countries. The PCR-micro plate hybridization assay was also sensitive enough to detect as little as 1pg of DNA; which is equivalent to approximately three bacilli. Nowadays, PCR could become a valuable alternative approach to the diagnosis of TB infections. Recently, a commercial PCR amplification Kit for the detection and identification of *M. tuberculosis* complex bacteria has became available. The target for the PCR is the 16 S rRNA sequence. The detection system is based on hybridization with *M. tuberculosis* complex specific capture probe in a micro plate format (Bloom, 1994).

**ii. Transcription mediated amplification (TMA)**

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

**iii. Strand displacement amplification (SDA)**

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

**c. Tuberculostearic Acid Test (TBSA)**

One easily detected components of *M. tuberculosis* is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography (Brooks et al., 1991). The presence of tuberculostearic acid in cerebrospinal fluid is thought to be diagnostic for tuberculous meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis. However, an important concern with pulmonary specimens is that

organisms other than *M. tuberculosis* may produce components that will generate a false positive signal (Bloom, 1994).

# CHAPTER-IV

## 4. MATERIALS AND METHODS

### 4.1 MATERIALS

A list of materials, chemicals, biological media and reagents required for this study is present in Appendix no. I and II.

### 4.2 METHODOLOGY

#### 4.2.1 Settings

This study was conducted in National Tuberculosis Centre, Bhaktapur, Nepal from November 2010 to April 2011.

#### 4.2.2 Type of study

Hospital based cross- sectional study.

#### 4.2.3 Inclusion criteria

- All newly diagnosed patients.
- Individuals diagnosed as MDR of less than 2 months and relapse cases

#### 4.2.4 Exclusion criteria

- Individuals known to be MDR (more than 2 month), XDR and defaulter tuberculosis patients.
- Individuals known to be HIV and HBs ag +ve.
- Patients with disseminated tuberculosis.
- Individuals with other known underlying diseases associated with infectious one.

#### 4.2.5 Sample size

A single early morning sputum sample from each 230 patients was evaluated.

#### 4.2.6 Collection of sputum sample

The sputum sample was collected in transparent, plastic, sterile, leak proof, screw capped disposable and appropriately labeled wide mouthed container. The container was labeled and filled in a request form available in the hospital. The specimen was kept at 4°C. The patients were instructed to collect 4 to 5 ml deep cough sputum, not saliva nor nasal secretions.

When sputum specimen was being collected adequate safety precautions were taken to prevent the spread of infectious organisms. Specimens collected should not be exposed to heat and light as tubercle bacilli are highly sensitive.

**4.2.7 Evaluation of the sputum sample**

A good sputum sample consists of recently discharged material from the bronchial tree with minimum amounts of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume. The specimen collected was processed within 2 hours or if not processed kept at 4°C. The specimen collected was stained by Ziehl-Neelsen stain using carbol fuchsin of 1%, 0.5% and 0.3% concentration and the sample was finally subjected to culture. When the specimen was mostly saliva, the specimen was reported as “unsuitable” for microbiological investigation and requested for another specimen.

**4.2.8 Sample processing**

The collected sputum sample was proceeded for microscopy and culture on the same day of collection

**a. Sputum smear microscopy**

Three direct smear was made from each sputum sample to stain with 3 different concentrations of carbol fuchsin.

**i. Ziehl - Neelsen method**

**Preparation of smear**

1. The slide was labeled with specimen number and date by diamond pencil.
2. A small portion of sputum (most purulent, most bloody, and most mucoid) was picked.
3. It was spread over the slide evenly and smear size of about 2×3 cm was made.
4. It was then dried at room temperature completely.
5. It was heat fixed by passing through the flame 2-3 times, spending for 5 seconds each time without scratching.

**Staining procedure:**

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

2. The stain was heated until vapour just began to rise (i.e. about 60°C).
3. The stain was allowed to remain in the slide for 5 minutes.
4. The stain was washed off with clean water.
5. The smear was decolorized by covering the smear with 25% sulphuric acid for 5 minutes or until the smear was sufficiently decolorized i.e. pale pink.
6. The slide was washed off with clean water.
7. The smear was covered with 0.1% methylene blue for 30S-60S.
8. The stain was washed off with clean water.
9. The back of the slide was cleaned and placed in a draining rack for the smear to air dry.

**Microscopic examination**

1. One drop of oil immersion was put on the left side of the stained smear.
2. The smear was observed first with 40x objective then with 100x power objective.
3. The examined slides were dipped into xylol to remove the oil immersion at the end of the examination.

**(Same staining procedure was repeated for 0.5% and 0.3% carbol fuchsin stain in the primary staining process).**

**ii. 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 senior technician. In addition all smears were independently reported by same senior technician and results were tallied before reporting. All reagent preparation was done by standard procedure (Appendix-III). All reagents were prepared by using distilled water to avoid false positive results (Collins et al.,1997).

**Recording and reporting**

If any definite red bacilli were seen, the smear was reported as AFB positive and reported according to the guidelines given by IUATLD.

| **Number of bacilli in smear** | **Results reported** |
|---|---|
| No AFB per 300 oil immersion field | 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 patient as well as the severity of tuberculosis diseases.

**b. Culture**

**i. Pretreatment**

1. About 2 volumes of 4% NaOH was added to one volume of sputum specimen. Then the cap of the container was tightened and shaken to digest and let stand for 15 minutes at room temperature.
2. The specimen was centrifuged at 3000xg for 15 minutes.
3. After centrifugation supernatant was discarded and distilled water was added and again centrifuged at 3000xg for 15 minutes.
4. Supernatant was decanted. The re-suspended sediment was inoculated to LJ media.

**ii. Inoculation and incubation**

One drop of sediment from homogenized and decontaminated sample was inoculated on two culture tubes of LJ medium. The caps of inoculated media were kept loose and incubated at 37°C. When the surface of media became dry, the caps were tighten and further incubated at 37°C until growth was observed or discarded as negative after 8 weeks.

**iii. Culture examination and observation**

All the cultured tubes were examined after one week to detect rapidly growing *Mycobacterium* which may be mistaken for *M. tuberculosis*. The growth on the media was observed at every week after inoculation. The negative cultures were discarded only after eight weeks. Typical colonies of *M. tuberculosis* were rough, tough, crumbly, waxy non-pigmented (buff colored) and slow-growers (growth appeared after 2-3 weeks after

inoculation) (Cheesebrough, 2000). Growth of *Mycobacterium* was confirmed by typical colony morphology, microscopy for AFB and different biochemical tests.

**iv. Recording and reporting of culture results**

The growth of AFB and contamination rate was recorded according to WHO guidelines as follows.

| | |
|---|---|
| (--) | : No growth |
| Actual figure | : 1-19 colonies |
| (+) | : 20-100 colonies |
| (++) | : 100-200 colonies |
| (+++) | : 200-500 colonies, almost confluent growth |
| (++++) | : More than 500 colonies, confluent growth. |

**Contamination rate**

C1+ =1/4 of the medium is contaminated

C2+ =1/2 of the medium is contaminated

C3+ =3/4 of the medium is contaminated

C4+ =Entire surface of the medium is contaminated

LQ =the medium is liquefied

**4.2.9 Biochemical identification of isolates**

**a. Niacin test**

Niacin test for the identification of *M. tuberculosis*

**Procedure**

1. About 1ml of sterile water was added to the culture slant. If the growth was confluent, the medium was punctured with pasture pipette to allow contact of water with the medium.
2. The tubes were placed horizontally for 30 minutes so that the fluid covers the entire surface of the medium for the extraction of niacin.

3. The extraction time may be longer if the culture has few colonies.
4. The slants were raised for 5 minutes to allow the fluid to drain to the bottom.
5. About 0.5 ml of the fluid extract was removed to a clean screw capped tube.
6. Sequentially 0.5 ml of the 4% alcoholic aniline solution and 0.5 ml of 10% aq. cyanogen bromide solution was added.
7. The tubes was closed and the solution was observed for the formation of a yellow colour (=positive result) within 5 minutes. The yellow colour appears as a ring at the interface of the two reagents, or if the tube is shaken, as a yellow column of liquid.
8. Finally, 2-3 ml of 4% NaOH was added to each tube and discarded.

***Results and interpretation**

# Negative: No colour

# Positive: Yellow colour appearing within 5 minutes. The colour appears as a ring at the interface of two reagents, or if the tube is shaken, as a yellow column of liquid.

**b. Nitrate reduction test**

Nitrate reduction test for identification of *M. tuberculosis.*

**Procedure**

1. About 0.2 ml of sterile saline was added to a screw-cap tube.
2. A sterile loop was used to emulsify 2 loopfulls of a 4 week old culture in the saline.
3. Then 2 ml of $NaNO_3$ substrate was added to it.
4. It was shaked well and incubated in a 37ºC water bath for 3 hours and removed.
5. The reagents were added in following order:

   a. 1 drop diluted HCL
   b. 2 drops 0.2% sulfanilamide
   c. 2 drops 0.1% N-naphthyethylene-diamine

6. It was examined immediately for the formation of pink to red colour and compared to the standard.

***Results and interpretation**

# Negative: No color. If no color develops, the test is either negative or the reduction has proceeded beyond nitrite. A small amount of powdered zinc was added to all negative tests by tipping the end of a slightly moistened applicator stick into dry zinc and shaking into the liquid.

i. If nitrate is still present, it will be catalysed by the zinc and a red colour will develop, indicating a true negative
ii. If no colour develops, the original reaction was positive but the nitrate was reduced beyond nitrite. The test was repeated to confirm the observation.

# Positive: Red colour, which vary from pink to very deep red-crimpson:

- **Faint pink = +/-**
- **Clear pink = 1+**
- **Deep pink = 2+**
- **Red = 3+**
- **Deep red = 4+**
- **Purplish red = 5+**

Only 3+ to 5+ is considered positive.

**c. Catalase test**

Heat labile test (68ºC, pH 7.0) for identification of *M. tuberculosis.*

**Procedure**

1. With a sterile pipette, aseptically 0.5 ml of 0.067M phosphate buffer, pH 7.0 was kept in screw cap tubes.

2. Several loopfulls of test cultures was suspended in the buffer solution using sterile loops.
3. The tubes containing the emulsified cultures were placed in a previously heated water bath at 68ºC for 20 minutes. Time and temperature was critical.
4. The tubes were removed from the heat and allowed to cool to room temperature.
5. Then 0.5 ml of freshly prepared Tween-peroxide was added to each tube and caps were replaced loosely.
6. Then it was observed for the formation of bubbles appearing on the surface of the liquid. The tubes were not shaken as Tween 80 may form bubbles when shaken, resulting in false positive results.
7. The negative tubes were held for 20 minutes before discarding.

**Results and interpretation**

# Positive: Bubbles

# Negative: No bubble

**Reporting TB cases**

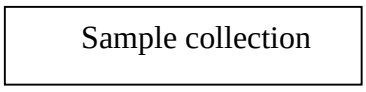
A patient was considered as a "TB- positive subject" if the sputum specimen had a positive culture and as a "non-TB subject" if the sputum specimen showed no growth.

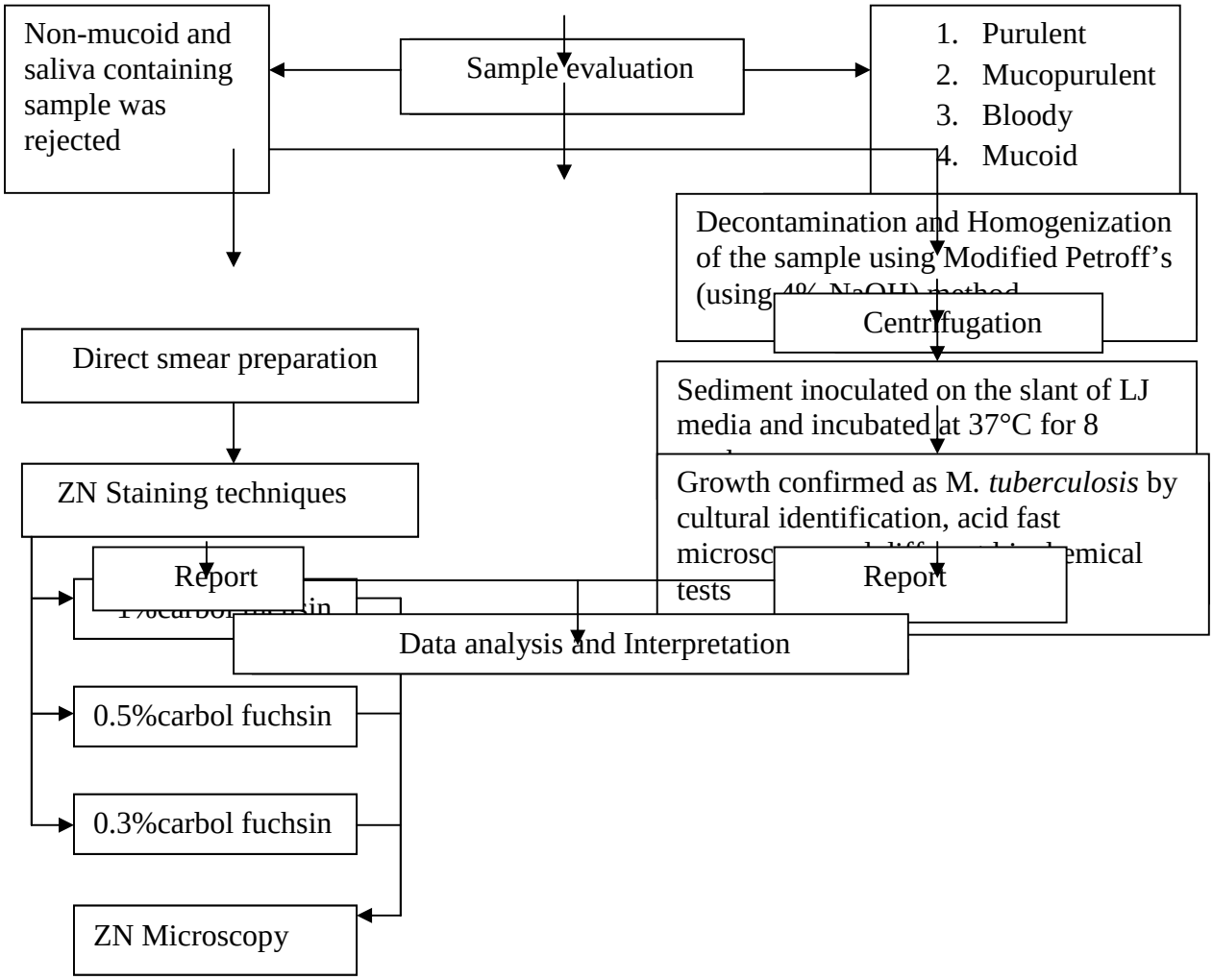
**4.2.10 Statistical analysis**

For the comparison of ZN staining techniques using different concentrations of carbol fuchsin, the statistical analysis of tests were carried out by calculating sensitivity, specificity, positive and negative predictive values, percentage of false negative and percentage of false positive (Appendix -VI). The McNemar's tests were calculated using statistical package for social services (SPSS) software version 11.5.

## 4.3 Flowchart of methodology and sample analysis

The experiment was designed to evaluate ZN staining techniques using 0.3%, 0.5% and 1% carbol fuchsin for detection of AFB in direct sputum sample.




Non-mucoid and saliva containing sample was rejected
Sample evaluation
1. Purulent
2. Mucopurulent
3. Bloody
4. Mucoid
Decontamination and Homogenization of the sample using Modified Petroff's
Centrifugation
Direct smear preparation
Sediment inoculated on the slant of LJ media and incubated at 37°C for 8
ZN Staining techniques
Growth confirmed as M. tuberculosis by cultural identification, acid fast
tests
Report
Report
Data analysis and Interpretation
0.5%carbol fuchsin
0.3%carbol fuchsin
ZN Microscopy

# CHAPTER -V

## 5. RESULTS

During the study period, a total of 230 Pulmonary tuberculosis suspected patients of different category attending National Tuberculosis Centre (NTC), were included. Among 230 patients, 148 were newly diagnosed, 42 were relapse cases, 13 were follow up, 6 were chronic and 21 were MDR (below 1 month) cases of tuberculosis. The sputum samples from patients under study were processed for the ZN staining using different concentrations of carbol fuchsin and the sputum sample was also processed for culture.

### 5.1 Age and sex wise distribution of PTB suspected patients:

Out of 230 PTB suspects, 73.0% (n=168) were males and 26.7% (n=62) were females in the age group of 10 to 80 years. The study showed the maximum number of patients visiting the NTC, Thimi belonged to the age group 21-30 (29.6%) and 31-40 (23.9%), followed by 51-60 (14.3%), 10-20 (13.9%), 41-50 (13.5%), 61-70 (3.9%) and 71-80 (0.9%).

**Table 1:** Age and sex wise distribution of PTB cases:

| Age group (years) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| 10-20 | 22 | 9.6 | 10 | 4.3 | 32 | 13.9 |
| 21-30 | 47 | 20.4 | 21 | 9.1 | 68 | 29.6 |
| 31-40 | 40 | 17.4 | 15 | 6.5 | 55 | 23.9 |
| 41-50 | 24 | 10.4 | 7 | 3.0 | 31 | 13.5 |
| 51-60 | 26 | 11.3 | 7 | 3.0 | 33 | 14.3 |
| 61-70 | 8 | 3.5 | 1 | 0.4 | 9 | 3.9 |
| 71-80 | 1 | 0.4 | 1 | 0.4 | 2 | 0.9 |
| Total | 168 | 73.0 | 62 | 26.7 | 230 | 100 |

### 5.2 Age and sex wise distribution of PTB confirmed patients:

Out of 230 sputum samples cultured, 65.65% (n=151) were culture positive, 27.83% (n=64) were culture negative and 6.52% (n=15) showed contamination. The contaminated culture results were excluded from the study for the examination of the staining techniques.

The gender wise distribution of TB showed more males were infected than females in all age groups. Out of the total culture positive isolates, 78.14% (n=118) were male and

21.83% (n=33) were female. The highest number of culture positive cases belonged to the age group 21-30 (30.45%), followed by 31-40 (23.76%), 10-20 (15.23%), 41-50 (15.22%), 51-60 (11.25%), 61-70 (3.31%) and 71-80 (0.66%).

**Table 2:** Distribution of culture positive isolates:

| Age group (years) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| 10-20 | 18 | 11.92 | 5 | 3.31 | 23 | 15.23 |
| 21-30 | 33 | 21.85 | 13 | 8.60 | 46 | 30.45 |
| 31-40 | 27 | 17.8 | 9 | 5.96 | 36 | 23.76 |
| 41-50 | 21 | 13.9 | 2 | 1.32 | 23 | 15.22 |
| 51-60 | 14 | 9.27 | 3 | 1.98 | 17 | 11.25 |
| 61-70 | 5 | 3.31 | 0 | 0 | 5 | 3.31 |
| 71-80 | 0 | 0 | 1 | 0.60 | 1 | 0.66 |
| Total | 118 | 78.14 | 33 | 21.83 | 151 | 100 |

### 5.3 Evaluation of staining techniques using different concentrations of carbol fuchsin and culture result:

The present study evaluated ZN staining techniques using different concentrations of carbol fuchsin (1%, 0.5% and 0.3%) to detect AFB in direct sputum smears. The staining methods were evaluated with culture on LJ medium, employed as ‘gold standard‘. The validities of the ZN staining methods using culture as the reference gold standard for the detection of AFB in direct sputum smears were determined.

The total positive results obtained by ZN staining technique using 1% carbol fuchsin was 125 (58.13%) and out of which the true positive (TP) was found to be 116 and false positive (FP) was found to be 9 (14.06%) and 90 (41.86%) as total negative, out of which 55 cases were true negative (TN) and 35 (23.17%) of them were false negative (FN). The sensitivity of ZN staining using 1% carbol fuchsin was found to be 76.82% and that of specificity was found to be 85.93%. Positive predictive values (PPV) and negative predictive values (NPV) were found to be 92.8% and 61.11% respectively.

**Table 3:** Evaluation of ZN staining using 1% carbol fuchsin with culture result:

| **ZN staining using 1% carbol fuchsin** | **Culture result** | | | **Sensitivity %** | **Specificity %** | **Predictive value** | |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | Total | | | Positive % | Negative % |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Positive | 116 | 9 | 125 | | | | |
| Negative | 35 | 55 | 90 | 76.82 | 85.93 | 92.8 | 61.11 |
| Total | 151 | 64 | 215 | | | | |

*P<0.05 for differences of ZN staining using 1% carbol fuchsin and culture by McNemar's test (P values= 0.000)

The total positive results obtained by ZN staining techniques using 0.5% carbol fuchsin were 118 (54.88%) and out of which the true positive (TP) was found to be 108 and false positive (FP) was found 10 (15.62%) and 97 as total negative, out of which 54 cases were true negative (TN) and 43 (28.47%) of them were false negative (FN). The sensitivity of ZN stain at 0.5% was found to be 71.52% and that of specificity was found to be 84.37%. Positive predictive values (PPV) and negative predictive values (NPV) were found to be 91.52% and 55.67% respectively.

**Table 4:** Evaluation of ZN staining using 0.5% carbol fuchsin with culture result:

| **ZN staining using 0.5% carbol fuchsin** | **Culture result** | | | **Sensitivity %** | **Specificity %** | **Predictive value** | |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | Total | | | Positive % | Negative % |
| Positive | 108 | 10 | 118 | | | | |
| Negative | 43 | 54 | 97 | 71.52 | 84.37 | 91.52 | 55.67 |
| Total | 151 | 64 | 215 | | | | |

*P< 0.05 for differences of ZN staining using 0.5% carbol fuchsin and culture by McNemar test (P values = 0.000)

The total positive result obtained by ZN staining technique using 0.3% carbol fuchsin were 103 (47.90%) and out of which the true positive (TP) was found 93 and false positive was found to be 10 (15.62%) and 112 as total negative (TN), out of which 54 cases were true negative (TN) and the 58 (38.41%) of them were false negative (FN). The sensitivity of ZN stain at 0.3% was found to be 61.58% and that of specificity was found to be 84.37%. Positive predictive values (PPV) and negative predictive values (NPV) were found to be 90.29% and 48.21% respectively.

**Table 5:** Evaluation of ZN staining Using 0.3% carbol fuchsin with culture result:

| **ZN staining using 0.3% carbol fuchsin** | **Culture result** | | | **Sensitivity %** | **Specificity %** | **Predictive value** | |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | Total | | | Positive % | Negative % |
| Positive | 93 | 10 | 103 | | | | |
| Negative | 58 | 54 | 112 | 61.58 | 84.37 | 90.29 | 48.21 |
| Total | 151 | 64 | 215 | | | | |

*P < 0.05 for differences of ZN staining at using 0.3% carbol fuchsin and culture by McNemar's test (P values = 0.000)

**5.4 False positive, False negative and True positive results obtained in different ZN staining techniques**:

The FP results produced by ZN staining using 1%, 0.5% and 0.3% carbol fuchsin were 14.06%, 15.62% and 15.62% respectively.

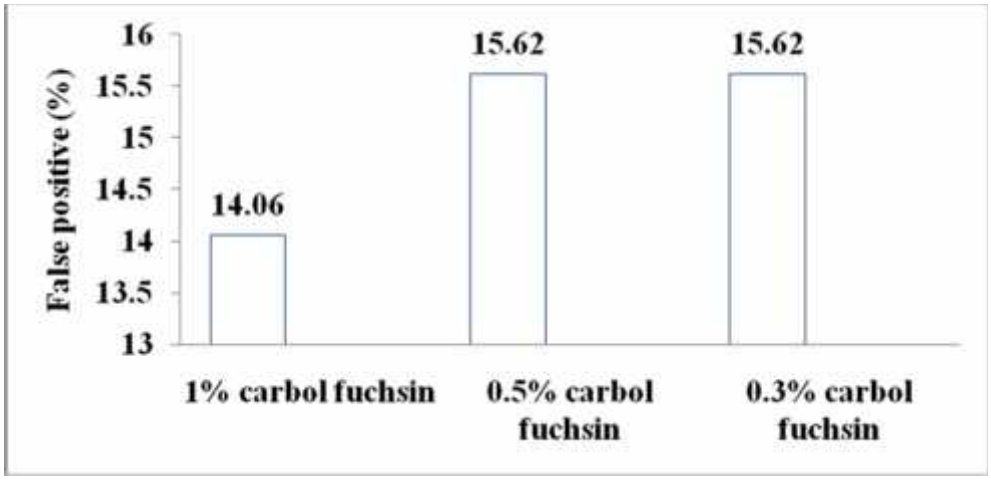



Figure 1: False positive Result obtained in different ZN staining techniques

The FN results produced by ZN staining using 1%, 0.5% and 0.3% carbol fuchsin were 23.17%, 28.47% and 38.41% respectively.

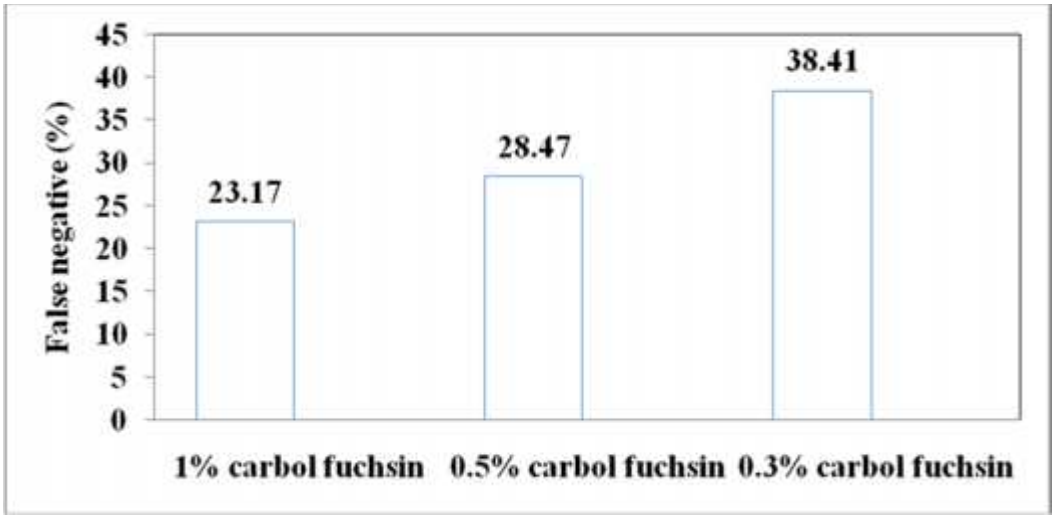



Figure 2: False negative Result obtained in different ZN staining techniques

The true positive cases obtained by ZN staining techniques using 1% carbol fuchsin showed highest result(116 out of 125) than at 0.5% carbol fuchsin (108 out of 118) and 0.3% carbol fuchsin (93 out of 103) (figure 3).

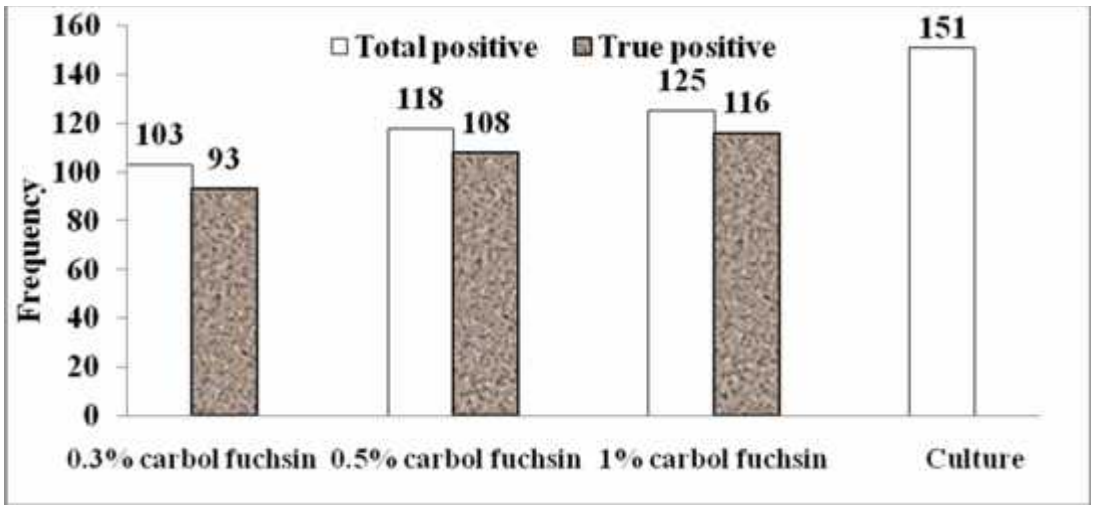


Figure 3: True Positive result obtained in different ZN staining techniques with reference to culture.

**5.5 Overall Correlation of different concentrations of carbol fuchsin in ZN staining techniques:**

The overall correlation of the ZN staining using 1% (79.53%), 0.5% (75.34%) and 0.3% (68.37%) carbol fuchsin showed that 1% carbol fuchsin has higher correlation with the culture as gold standard than 0.5% and 0.3% carbol fuchsin.

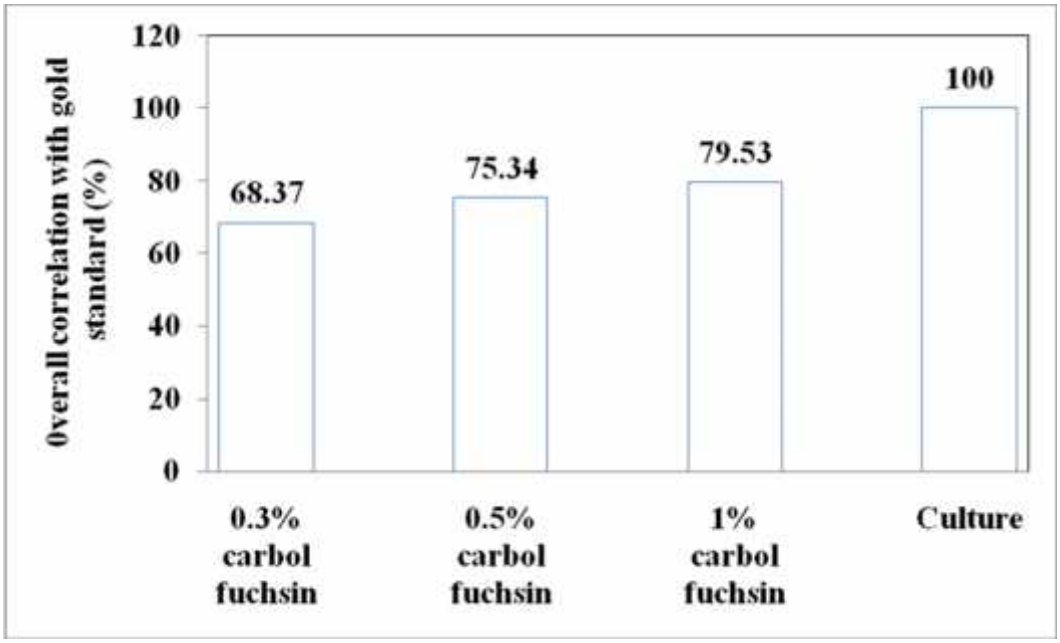


Figure 4: Overall correlations of the ZN staining techniques using 1%, 0.5% and 0.3% carbol fuchsin with the culture.

**5.6 Correlation of ZN staining techniques using 1%, 0.5% and 0.3% carbol fuchsin:**

In this present study 230 sputum samples were examined parallel for the correlation between ZN staining technique using 1%, 0.5% and 0.3% carbol fuchsin. The positive

agreement in between 1% and 0.5% was 89.23% whereas positive agreement in between 1% and 0.3% was 79.23%. 6 (6%) cases which were not detected by 1% were detected by 0.5% (Table 6). The total yield of positive result by ZN staining using 1% carbol fuchsin was 130 (56.52%) as compared to 122 (53.04%) and 107 (46.52%) found by using 0.5% and 0.3% carbol fuchsin respectively.

**Table 6:** Correlation between ZN staining techniques using 0.5% and 1% carbol fuchsin in slide reading of AFB smear Positive and negative:

| **ZN staining using 0.5% Carbol fuchsin % (n)** | **ZN staining using 1% carbol fuchsin % (n)** | | **Total % (n)** |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 89.23 (116) | 6 (6) | 53.04 (122) |
| Negative | 10.76 (14) | 94 (94) | 46.95 (108) |
| **Total** | 100 (130) | 100(100) | 100 (230) |

* P > 0.05 for differences for ZN staining using 0.5% and 1% carbol fuchsin by McNemar's test (P values = 0.115).

**Table 7:** Correlation between ZN staining techniques using 0.3% and 1% carbol fuchsin in slide reading of AFB smear positive and negative:

| **ZN staining using 0.3% Carbol fuchsin % (n)** | **ZN staining using 1% carbol fuchsin % (n)** | | **Total % (n)** |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 79.23 (103) | 4 (4) | 46.52 (107) |
| Negative | 20.76 (27) | 96 (96) | 53.47 (123) |
| **Total** | 100(130) | 100(100) | 100 (230) |

*P < 0.05 for differences for ZN staining using 0.3% and 1% carbol fuchsin by McNemar's test (P values = 0.000)

The quantitative comparison of smears for AFB stained using 1%, 0.5% and 0.3% carbol fuchsin was also done.

**5.7 Quantitative comparison of smears made using 0.5% and 1% carbol fuchsin:**

Of the 116 positive smears by both methods, 104 positive smears had equal number of AFB on both smears, 11 positive smears had more AFB on the smear stained using 1% carbol fuchsin and 1 positive smear had greater number of AFB on the smear stained using 0.5% carbol fuchsin. Moreover, 14 cases shown negative by 0.5% carbol fuchsin was shown positive by 1% carbol fuchsin and only 6 cases shown positive by 0.5% were missed by 1% carbol fuchsin. It was found that more scanty (positive) cases shown by 1% carbol fuchsin were missed by 0.5% carbol fuchsin

**Table 8:** Quantitative comparison of sputum smears for AFB detection stained using 1% carbol fuchsin and 0.5% carbol fuchsin:

| **Carbol fuchsin (0.5%)** | **Carbol fuchsin (1%)** | | | | | **Total** |
|---|---|---|---|---|---|---|
| | Negative | 1+ | 2+ | 3+ | scanty | |
| Negative | 94 | 4 | 0 | 3 | 7 | 108 |
| 1+ | 1 | 37 | 1 | 0 | 0 | 39 |
| 2+ | 1 | 1 | 28 | 2 | 0 | 32 |
| 3+ | 2 | 0 | 0 | 35 | 0 | 37 |
| Scanty | 2 | 7 | 1 | 0 | 4 | 14 |
| **Total** | 100 | 49 | 30 | 40 | 11 | 230 |

* P > 0.05 for differences of quantitative comparison of stained using 0.5% and 1% carbol fuchsin (P values=0.46)

**5.8 Quantitative comparison of smears made using 0.3% and 1% carbol fuchsin:**

Of 103 positive smears by both methods, 67 positive smears had equal number of AFB on both smears, 35 positive smear had more AFB on the smear stained using 1% carbol fuchsin and 1 positive smear had more AFB on the smear stained using 0.3% carbol fuchsin. Moreover, 27 smear positive cases by 1% carbol fuchsin was shown negative by

0.3% carbol fuchsin and 4 negative cases of 1% carbol fuchsin was reported as positive by 0.3% carbol fuchsin. It was found that more scanty (positive) cases shown by smear stained by 1% carbol fuchsin was found negative with smear stained with 0.3% carbol fuchsin.

**Table 9:** Quantitative comparison of sputum smears for AFB detection stained using 1% carbol fuchsin and 0.3% carbol fuchsin:

| **Carbol fuchsin (0.3%)** | **Carbol fuchsin (1%)** | | | | | **Total** |
|---|---|---|---|---|---|---|
| | Negative | 1+ | 2+ | 3+ | scanty | |
| Negative | 96 | 14 | 0 | 3 | 10 | 123 |
| 1+ | 0 | 22 | 13 | 1 | 0 | 36 |
| 2+ | 1 | 1 | 15 | 7 | 0 | 24 |
| 3+ | 0 | 0 | 0 | 29 | 0 | 29 |
| Scanty | 3 | 12 | 2 | 0 | 1 | 18 |
| **Total** | 100 | 49 | 30 | 40 | 11 | 230 |

* P < 0.05 for differences of quantitative comparison of smear stained using 0.3% and 1% carbol fuchsin by McNemar's test (P values=0.000).

### 5.9 Overall comparison of smear results using different concentrations of carbol fuchsin with culture results:

The total 3+, 2+, 1+, scanty and negative cases found by smear stained with 1% carbol fuchsin was found to be 42, 28, 45, 15 and 100 respectively. Similarly the smear stained with 0.5% showed 35, 32, 38, 17 and 108 cases of 3+, 2+, 1+, scanty and negative. And that of 0.3% was found to be 29, 26, 33, 19 and 123 for 3+, 2+, and 1+, scanty and negative respectively. These all results were compared with that of culture result whose values for 3+, 2+, 1+, scanty and negative were 84, 32, 29, 9, 64 respectively and 15 cases were the contaminated cases. The proportion of smear negative but culture positive

specimens was higher with the modified –ZN (0.3%) method than with 0.5% and standard ZN (1%) method.

**Table: 10** Comparison of smear results of modified – ZN method, 0.5% method and standard - ZN method with culture results:

| **Method** | **Smear result** | **No. with culture result** | | | | | | **Total** |
|---|---|---|---|---|---|---|---|---|
| | | 3+ | 2+ | 1+ | Scanty | Negative | contamination | |
| Standard –ZN (1%) | 3+ | 28 | 8 | 2 | 2 | 0 | 2 | 42 |
| | 2+ | 15 | 6 | 3 | 1 | 2 | 1 | 28 |
| | 1+ | 25 | 8 | 8 | 0 | 2 | 2 | 45 |
| | Scanty | 4 | 4 | 2 | 0 | 5 | 0 | 15 |
| | Negative | 9 | 6 | 14 | 6 | 55 | 10 | 100 |
| 0.5% method | 3+ | 22 | 8 | 2 | 2 | 0 | 1 | 35 |
| | 2+ | 14 | 8 | 3 | 1 | 5 | 1 | 32 |
| | 1+ | 22 | 6 | 4 | 0 | 4 | 2 | 38 |
| | Scanty | 5 | 5 | 6 | 0 | 1 | 0 | 17 |
| | Negative | 18 | 5 | 14 | 6 | 54 | 11 | 108 |
| Modified –ZN (0.3%) | 3+ | 20 | 5 | 2 | 1 | 0 | 1 | 29 |
| | 2+ | 10 | 8 | 2 | 2 | 3 | 1 | 26 |
| | 1+ | 17 | 7 | 4 | 0 | 3 | 2 | 33 |
| | Scanty | 8 | 3 | 4 | 0 | 4 | 0 | 19 |
| | Negative | 26 | 9 | 17 | 6 | 54 | 11 | 123 |
| | **Total** | 81 | 32 | 29 | 9 | 64 | 15 | 230 |

# CHAPTER – VI

## 6. DISCUSSION AND CONCLUSION

### 6.1 Discussion

Sputum smear microscopy is a simple and cost effective tool which provides not only a preliminary confirmation of the disease but also a quantitative estimate of the number of bacilli. Smear positivity correlates well with the severity of pulmonary disease, infectiousness, and risk of death if untreated. The ZN method for detection of AFB is easy, rapid, and inexpensive. Cultures are generally not performed in developing countries, and in any case they require several weeks to become positive. The quest for rapidity and efficacy has resulted in several modifications to simplify the ZN method. The approaches have included bulk staining of slides, staining of slides by using a microwave oven, use of a decalcifying agent, chloroform, carbol fuchsin-impregnated strips, and cold staining. However, none of these modified methods have gained wide acceptance and are not used. Recently, the WHO recommended the use of 0.3% carbol fuchsin. This study is based on comparison of 3 different concentrations of carbol fuchsin in detecting Acid fast bacilli.

In the present study, 230 sputum specimens from clinically suspected PTB patients were studied. During sample collection all the patients were advised to submit two early morning sputum samples from each patient similar to as Crampin et al. (2001) and Harries et al. (2004), who reported smear microscopy examination of 2 spontaneous sputum specimens is the most efficient and examining two sputum smears is sufficient for the early detection of AFB. Though WHO recommended 3 sputum samples (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 specimens.

In the study out of 230 patients, 151 (65.65%) were diagnosed as having PTB and the *M. tuberculosis* was isolated by conventional culture technique from sputum specimen. The growth of organism in culture as *Mycobacterium tuberculosis* was identified by cultural characteristics, acid-fast staining, and with biochemical tests as Nitrate test, Niacin test

and catalase test as heat labile test. However, Positive culture confirmed by microscopy and biochemical tests could not be tested with other nucleic acid amplification methods as PCR for further identification.

In the present study, maximum number of suspected patients of TB visiting the centre for the diagnosis was observed in the age group of 21- 30 (29.6%) and with more males (73.0%) than the females (26.7%). Considering culture positive result, TB cases were again observed in the age group of 21- 30 (30.45%). This kind of figure is very similar to entire developing world including Nepal as reported in the WHO report, 2006. Also the study conducted by Shrestha (2006) has reported increased incidence of tuberculosis in the age group of 15- 34 years where 66.97% of males were AFB positive. Similar results were reported by Bhatt et al., in 2009, Sapkota et al., in 2008; in Nepal and also by Mabaera et al., in 2006, and Finch and Beaty in 1997, in other country.

The gender wise consideration of TB positive cases showed more males (78.14%) were culture positive, as compared to females (21.83%). This does not however reflect an increase in the occurrence of diseases in males, since the attendance of females to OPD is lower than males. Also the high suspected cases of TB in males might be due to movement of males in different TB endemic and crowded area (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 tends to be more active and are more likely to interact with other people than the elderly and the young one. The finding is in accordance with the reports presented by Diwan and Thorson (1999); Chan-Yeung et al. (2002) and Khan et al. (2007) who reported lower smear positivity in women than in man.

From this study, TB incidence in children below 15 years was not found in the PTB suspects. This data is in accordance to the review done by Nelson and Wells (2004) and Kabra et al. (2004), who reported the diagnosis of TB in children is difficult, and rarely rests on bacteriologic confirmation though there is an increase in childhood tuberculosis in many parts of the world. So, for the diagnosis of childhood TB, assessment of optimal methods of contact investigations and an analysis of NTP data to assess risk factors for adverse outcomes (e.g. death, default treatment failure) among children is needed.

In the study the validity of ZN staining techniques using different concentration of carbol fuchsin was compared by using culture as gold standard for the detection of AFB in direct sputum smears. The sensitivity of ZN staining using different concentrations of carbol fuchsin i.e. 1%, 0.5% and 0.3% was found to be 76.82%, 71.52% and 61.58% respectively (Table 3, 4 and 5). The sensitivity difference is quite higher between 1% and 0.3% carbol fuchsin than in case of 1% and 0.5% carbol fuchsin. This data is in accordance to the study done by Selvakumar et al., 2002 who found a significant difference in sensitivity i.e. 83.6% in 1% carbol fuchsin and 72.1% in 0.3% carbol fuhsin. The study by Selvakumar et al., 2005 concluded that reducing concentration of basic fuchsin below 0.3% in ZN staining techniques was found to significantly reduce its sensitivity. According to the study, the sensitivity of ZN using 0.3% (65%) and 1% basic fuchsin (62%) was comparable while it was reduced using 0.1% (74%) compared to 1% basic fuchsin (83%). Also the 0.3% 5 minute ZN staining technique showed more false negatives and lower sensitivity than 1% 5 minute ZN staining (55, 86.5% vs. 42, 89.9%) without reaching statistical significance, in the study conducted by Deun et al in 2005.

Thus study was in contrast to the study of Deun et al., 2005 whose study showed that the sensitivity of the WHO/IUATLD recommended 0.3% carbol fuchsin for 5 minute was not significantly different from the original 1% ZN for 5 minute but the study found that 1% carbol fuchsin for 15 minute might be superior.

In the present study, the sensitivity of ZN staining technique using 1% carbol fuchsin (76.82%) was found somewhat comparable to 0.5% carbol fuchsin (71.52%) but was significantly higher than that of ZN staining at 0.3% carbol fuchsin (61.58%). Rickman and Mayer (1980) reported the increased sensitivity can be misleading as it might be accompanied by the decrease in true positive and increase in relative false positive cases.

The specificities of ZN staining techniques using 1%, 0.5% and 0.3% carbol fuchsin were found to be 85.93%, 84.37% and 84.37% respectively. The true negative rate was slightly higher in case of 1% carbol fuchsin (55 cases) whereas the rate was equivalent in case of 0.5% and 0.3% (54 cases) carbol fuchsin methods. This data is in accordance with the study done by Selvakumar et al., 2002 where the specificity of 1% and 0.3% carbol fuchsin was 93.6% and 95.5% respectively. There was very little difference in specificity of 1% and 0.3% carbol fuchsin as in our study. The specificity on smear examination

methods should be interpreted with caution as the artifacts might be mistaken for the bacilli that might reduce the specificity.

The false positive (FP) result in case of 1% carbol fuchsin was 14.06% and in case of 0.5% and 0.3% carbol fuchsin was 15.62% (Figure 1). The FP results in 0.5% and 0.3% carbol fuchsin are similar whereas it was slightly lower in case of 1% carbol fuchsin. The main reason for increase in FP may be due to dead bacilli, from patients who could have already been on treatment, as this study consists of relapse, MDR (below 1 month), follow-up and chronic cases. Among the patients who are not receiving chemotherapy, the important cause of false positive of smear microscopy might be due to *Mycobacterium* other than tuberculosis (MOTT), which does not grow in the selective media such as LJ media, and also due to use of overly harsh procedures for the specimen decontamination during sample processing for the culture. This might be true in this study as the contamination rates was relatively higher 6.52% (n=15). Also the false positive results suggest that occasionally a sputum specimen on a smear may contain particles that are acid fast: these particles may sometime resemble tubercle bacilli i.e. MOTT or the precipitates of staining, which hampers the reading (Shrestha et al., 2005).

The false negative (FN) rate of ZN staining technique using 1%, 0.5% and 0.3% carbol fuchsin was found 23.17%, 28.47% and 38.41% respectively (Figure 2). ZN staining using 0.3% carbol fuchsin has increased FN than ZN staining using 0.5% and 1% carbol fuchsin. False negative rate was somewhat comparable between 1% (23.17%) and 0.5% (28.47%) carbol fuchsin but FN rate of 0.3% was significantly higher than that of 1% carbol fuchsin. High FN rate might be due to deficiencies of enough material in the preparation of direct smear despite the use of particular staining techniques. Also it might be due to the technical limitation of direct sputum smear microscopy that between 5,000 and 10,000 tubercle bacilli per milliliter of sputum are required for direct microscopy to be positive and only a proportion of tuberculosis patients harbor large enough number of organisms to be detected in this way (WHO 1998 a). It is also virtually impossible to distinguish different mycobacterial species by microscopy. AFB might be missed if overly thick smears are prepared, decolourization is too vigorous or poorly contrasting counter stains are used. False negative result might also be due to high sensitivity of the culture as culture increases the number of TB cases found often by 30-50% as reported

by WHO, 1998 a since culture techniques can detect as few as 10 bacilli per milliliter of sputum.

In the present study, the true positive (TP) was found to be more 116 (92.8%) in the ZN staining using 1% carbol fuchsin than that of 0.5% (91.52%) and 0.3% (90.25%) carbol fuchsin. The true positive rate was comparable between 1% and 0.5% carbol fuchsin but the rate was higher in case of 1% than that of 0.3% carbol fuchsin (Figure 3).

The overall correlation of ZN staining techniques using 1%, 0.5% and 0.3% carbol fuchsin was found 79.53%, 75.34% and 68.37% respectively in the study. The overall correlation indicate the chance of TP in the 1% carbol fuchsin ZN staining technique is higher than that of 0.5% and 0.3% carbol fuchsin ZN staining technique. There was significantly high difference in overall correlation in between 0.3% and 1% carbol fuchsin ZN staining technique. Urbanczik (1985) reported two main reasons for the change in overall correlation of positive smears to the culture results as the epidemiological and the technical such as number and type of specimen for the diagnosis of TB.

In the present study, the Positive predictive value of 1%, 0.5% and 0.3% carbol fuchsin were 92.8%, 91.52% and 90.29% respectively whereas Negative predictive value of 1%, 0.5% and 0.3% carbol fuchsin were 61.11%, 55.67% and 48.21% respectively. The higher predictive value in case of 1% carbol fuchsin is due to higher rate of true positives than in case of 0.3% carbol fuchsin. Also high negative predictive value in case of 1% carbol fuchsin is due to higher true negative cases and less total negative cases. The above data suggests that 1% carbol fuchsin shows the higher amount of true negative, true positive cases and fewer amounts of false negative and false positive cases.

The correlation between 0.5% carbol fuchsin and 1% carbol fuchsin with the positive agreement in between both the technique was 89.23% (n=116) (Table 6 and 8) whereas positive agreement between 1% and 0.3% carbol fuchsin was 79.23% (n=103) (Table 7 and 9). This agreement was 10% less than the agreement between 1% and 0.5% carbol fuchsin. The ZN staining using 1% carbol fuchsin increases the smear positivity by 10.7% over 0.5% carbol fuchsin and 20.76% over 0.3% carbol fuchsin. Of the 116 positive smears by both staining methods, 104 (89.65%) had equal numbers of AFB on

both smears, 12 (10.34%) had more AFB on the smear stained using 1% carbol fuchsin and 1 (0.865%) had more AFB on the smear stained using 0.5% carbol fuchsin (Table 8). Also among 103 positive smears by both staining methods, 67 (65.04%) had equal numbers of AFB on both smears stained using 1% and 0.3% carbol fuchsin, 35 (33.9%) had more AFB on the smear stained using 1% carbol fuchsin and 1 (0.97%) had more AFB on the smear stained using 0.3% carbol fuchsin (Table 9). In both the cases i.e. 0.5% and 0.3% carbol fuchsin, only 1 more positive case than 1% carbol fuchsin was found. This results supports the increased sensitivity of the ZN staining using 1% carbol fuchsin..

The quantitative comparison of all three methods of ZN staining was done with culture where each of 9, 18 and 26 negative cases shown by 1%, 0.5% and 0.3% carbol fuchsin respectively showed higher density of AFB by culture. The data also suggests that low cases i.e. only 9 cases which were positive by culture were missed by 1% carbol fuchsin but the missing rate was maximum in 0.3% carbol fuchsin i. e. 26.

**6.2 Conclusion**

The present study is done to evaluate three different concentrations of carbol fuchsin in ZN staining techniques used in the primary diagnosis of pulmonary TB. Today, attention has turned to nucleic acid technology: the PCR and related techniques are rapid, specific and sensitive. However these methods require more sophisticated laboratory methods and are not being used for the routine diagnosis of TB. Detection of AFB by sputum smear microscopy is the only feasible method recommended for the tuberculosis control program in Nepal and many other developing countries in detecting infectious pulmonary tuberculosis cases and for monitoring the progress of patients during treatment.

ZN microscopy is the most common method followed in the laboratory for detection of AFB. The present study was done to evaluate the three concentrations (1% 0.5% and 0.3% carbol fuchsin) of carbol fuchsin in detecting AFB. 1% carbol fuchsin was overall more correlated to culture and was with more positive agreement with culture. It had more sensitivity and specificity than 0.5% and 0.3% carbol fuchsin. It was more significant than 0.5% and 0.3% carbol fuchsin. Moreover, 1% carbol fuchsin showed more true positive cases and less false negative and false positive cases. Hence, the study

concludes that the diagnosis of PTB could be made by 1% carbol fuchsin ZN staining technique (ZN microscopy).

# CHAPTER -VII

## SUMMARY AND RECOMMENDATIONS

### 7.1 Summary

The major findings of the study are summarized as follows:

1. Out of 230 PTB suspected patients, 78.14% male and 21.83% female were suffered from TB. Gender wise consideration of TB showed more males were found to be infected than females in all age groups.
2. Maximum numbers of TB cases were observed in the age group of 21-30 years (30.45%). Pulmonary tuberculosis was not diagnosed in the cases below 10 years.
3. One hundred fifty one (65.65%) patients out of 230 were found AFB positive by culture and the contamination rate was 6.25%. The positive results by 1%, 0.5% and 0.3% carbol fuchsin were 58.13%, 54.88% and 47.9% respectively.
4. The sensitivity of ZN staining using 1%, 0.5% and 0.3% carbol fuchsin was 76.82%, 71.52% and 61.58% respectively.
5. The Specificity was found to be 85.93% in 1% carbol fuchsin and 84.37% in 0.5% and 0.3% carbol fuchsin.
6. The positive predictive value in different ZN staining technique using 1% Carbol fuchsin, 0.5% Carbol fuschin and 0.3% carbol fuchsin was 92.8%, 91.52% and 90.29% respectively.
7. The negative predictive value in ZN staining techniques using 1% carbol fuchsin was 61.11%, 0.5% carbol fuchsin was 55.67% and 0.3% carbol fuchsin was 48.21%.
8. The false positive result obtained in the study was 14.06% for 1% carbol fuchsin and 15.62% for 0.5% and 0.3% carbol fuchsin.
9. The false negative results obtained for ZN staining techniques using 1% carbol fuchsin, 0.5% carbol fuchsin and 0.3% carbol fuchsin were 23.17%, 28.47% and 38.41% respectively.
10. The true positive cases of 1% carbol fuchsin, 0.5% carbol fuchsin and 0.3% carbol fuschin were 116 out of 125, 108 out of 118 and 93 out of 103 respectively.
11. When ZN staining using 0.5% carbol fuchsin was correlated with ZN staining using 1% carbol fuchsin, the positive agreement of 89.23% was found.

12. When ZN staining using 0.3% carbol fuchsin was correlated with ZN staining using 1% carbol fuchsin, the positive agreement of 79.23% was found.
13. Out of 116 AFB positive smears by both methods i.e.1% carbol fuchsin and 0.5% carbol fuchsin, 89.65% had equal numbers of AFB on both smears, 12 (10.34%) had more AFB on the smear stained using 1% carbol fuchsin and 1 (0.86%) had more AFB on the smear stained using 0.5% carbol fuchsin.
14. Out of the 103 AFB positive smears by both staining methods i.e.1% carbol fuchsin and 0.3% carbol fuchsin, 67 (65.04%) had equal numbers of AFB on both smear 35 (33.9%) had more AFB on the smear stained using 1% carbol fuchsin and 1 (0.97%) had more AFB on the smear stained using 0.3% carbol fuchsin.

Since, majority of smear positive with ZN staining using 1% carbol fuchsin were also positive by culture, from the present study, it can be concluded that ZN staining using 1% carbol fuchsin is more sensitive than the ZN staining using 0.5% and 0.3% carbol fuchsin

**Recommendations:**

1. 1% carbol fuchsin is recommended instead of 0.3% carbol fuchsin for smear microscopy in the laboratories.
2. Culture should always be requested if available, to reduce any error as false negative in the ZN microscopy.
3. ZN staining using 1% carbol fuchsin should be further evaluated for concentrated sputum smears.

4. Further study should be done for 1% and 0.5% carbol fuchsin with large number of samples.

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