CHAPTER- I

1. INTRODUCTION

If the number of victims which a disease claims is the measure of its significance, then all the disease, particularly the most dreaded infectious disease, such as bubonic plague, Asiatic cholera etc., most rank far behind tuberculosis

Robert Koch, 1882

Tuberculosis (TB) 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).

The history of sputum examination dates back to March 24, 1882 when Robert Koch discovered the tubercle bacillus and confirmed the bacterial etiology of TB (Ponticeellio *et al*., 2001). Clinical screening by assessment of symptoms identifies pulmonary TB (PTB) suspects among patients attending health facilities. Sputum smear microscopy is most effective method for screening PTB suspects in high- prevalence countries. Detection of smear positive cases identifies those cases that are infectious and contributing substantially to the transmission of disease (Harries *et al*., 1998). Acid-fast bacilli (AFB) microscopy which is a means of detecting and screening of PTB, has been used worldwide and it remains as a mainstay of case finding (Barez *et al*., 1995). The finding of AFB in sputum establishes a presumptive diagnosis of TB and is crucial to guide treatment, to limit person to person spread and to assess the degree of the activity of the disease (Behr *et al*., 1999).

The highest priority for TB control is given to the identification of patients with sputum smear–positive (SS+) PTB and cure of the infectious cases because this helps in the reduction of the mode of transmission thereby reducing overall mortality and morbidity (Harries *et al*., 1998; Kivihya *et al*., 2004).

Acid -fast staining technique is the fastest, easiest, and least expensive tool for the rapid identification of potentially infectious TB patients (Murray *et al*., 2003). The importance of the method is further exemplified by the fact that in low-income countries, the definitive diagnosis of TB still relies solely on the presence of AFB in stained smears. Though the specificity of acid-fast microscopy is excellent for mycobacterial species, the sensitivity is not optimal. The sensitivity of microscopy is influenced by numerous factors, such as the prevalence and severity of disease, the type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, the staining technique, and the quality of the examination. However, the specificity of smear examination methods should be interpreted with caution because it does not allow differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT).

Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One type is carbol fuchsin (Ziehl-Neelsen [ZN] or Kinyoun methods), and the other is fluorochrome either auramine phenol (AP) or auramine-rhodamine (AR). The present ZN method has evolved from Koch's original alkaline methylene blue-based method following significant modifications. Kinyoun cold staining is a modification of the classical ZN method that excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin (Somoskovi *et al*., 2001). There are several other modified techniques for staining AFB and one of these is Modified cold (MC) stain. MC stain is two steps cold staining method which requires concentrated carbol fuchsin as a primary stain and Gabbet methylene blue as counter stain (Bhat and Bhat, 2000). This simple staining has added advantage of non requirement of heat as well as a separate step for decolourisation and counter stain. Simple MC stain is a new, cheaper, safer, and easier cold staining method which is suitable for laboratories with minimum facilities and in setting where work load is heavy (Kochhar, 2002). Methods applying

fluorochrome have been used for acid-fast staining since the introduction of an auramine O and an AR based methods. ZN stain is commonly used throughout the world and still remains the standard method against which new tests must be measured. All techniques are based on the relatively unique property of *Mycobacterium* to retain the primary stain even after exposure to strong mineral acid or acid-alcohol, hence the term, acid-fast bacilli (Sonnenwirth and Jarett, 1990).

The smears stained by ZN method can detect bacilli when they are at the order of 10^5/milliliter (ml) of sputum, whereas a more sensitive staining technique like fluorescent stain detects the bacilli when they are at the order of 10^4/ml of sputum (Forbes *et al*., 1998; Katoch, 2004). Because it is easier to detect a fluorescent rod against a darker background, the fluorochrome staining method allows the examiner to scan the slide at a lower magnification and thus observe a larger area than with carbol fuchsin-stained smears. These factors reduce the time for screening and lead to greater sensitivity. Therefore, it is generally accepted that the fluorescent method should be given preference over the ZN and Kinyoun methods (Somoskovi *et al*., 2001).

The definitive diagnosis of TB depends on the isolation and identification of *M. tuberculosis*. The inoculation of concentrated bacilli from processed clinical specimens on solid media is a standard approach for confirmation of TB (Kar *et al*., 2003). Culture methods are more sensitive than microscopy as it can detect 10-100 mycobacteria per ml of sample and give positive result. Therefore culture is deemed to be the gold standard for diagnosis of TB (Grange, 1990). Despite its enhanced sensitivity and specificity, culture is of impractical clinical use, because it is costly, time consuming and requires specialized safety laboratories, which is usually not performed in most low income countries (Parekh and Kar, 2003).

Key factors in TB control are rapid detection, adequate therapy and contact tracing to arrest further transmission. The traditional diagnostic tools, apart from a thorough clinical examination, are chest X-ray, which is sensitive but not specific and use of serological tests and Polymerase chain reaction (PCR) are of unproven value in TB

control. Progress in molecular methods is restricted to only few sophisticated laboratories (Kochhar, 2002; Tanuphasiri and Kladphuang, 2002).

In developing countries like Nepal, TB laboratories' services are able to conduct sputum smear microscopy at provincial and district hospitals. Communities and health centers have minor roles in carrying out TB services because health workers have insufficient experience with diagnostic testing. Culture and sensitivity testing are available only at the central level. Therefore, under the present circumstances sputum microscopy is nevertheless a rapid way of detecting the most contagious patients, and its specificity is high. If the sensitivity could be increased it would be even more useful diagnostic tool in the developing world (Angeby *et al*., 2000). In sputum smear microscopy, ZN is the most commonly used technique, because of its simplicity and low cost. There are also other staining techniques for detection of acid fast bacilli, which are simpler, rapid and more sensitive than ZN. The present study evaluates four different staining techniques used in the detection of AFB. The comparative evaluation of different staining procedures used in sputum smear microscopy will help to know appropriate staining method for demonstration of AFB in laboratory and public set-up on the basis of sensitivity and specificity obtained in each staining technique with the reference to sputum culture.

CHAPTER - II

2. OBJECTIVES OF THE STUDY

2.1 GENERALOBJECTIVE

To evaluate different staining techniques (Ziehl-Neelsen stain, Kinyoun stain, Modified cold stain, and Fluorochrome stain) for the preliminary diagnosis of pulmonary tuberculosis.

2.2 SPECIFIC OBJECTIVES

i To evaluate four different staining techniques for detection of acid–fast bacilli (AFB) in sputum sample.

ii To determine most appropriate method of staining in a laboratory set up.

CHAPTER – III

3. LITERATURE REVIEW

Tuberculosis, a major public health problem is the most common cause of death due to a single infectious agent in adults and accounts for over a quarter of all avoidable deaths (Mohan and Sharma, 2001). TB infection is caused primarily by *Mycobacterium tuberculosis* and less commonly by *M. africanum* or *M. bovis* (Collee *et al*., 1996). This disease has the potential to infect virtually every organ, most importantly the lungs due to dissemination via lympho-hematogenous route (Haas, 2000).

3.1 EPIDEMIOLOGY

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

With 22% of global population, SAARC region bears 29% of global TB burden with approximately 2-5 million new cases and 0.6 million deaths per year. More than 1.1 million new persons develop infectious (SS+) pulmonary TB each year in this region (STC, 2003). In South Asia, India, Bangladesh and Pakistan have the largest number of TB patients. Amongst these countries, India carries the greatest burden of TB cases.

TB is one of the major public health problems in Nepal. According to WHO, Nepal is 27th highest TB burden country in the world, with estimated annual risk of infection of 1.8% (WHO, 2001). About 45% of the total population is infected with TB, out of which 60% are in adult age group. In Nepal, 80,000 people have TB. Every year about 40,000 people develop TB. Nearly half of them i.e. 20,000 have infectious sputum positive TB. It is estimated that about 10,000 people die from TB every year, *i.e*. 200 deaths every week, over 25 deaths every day (NTC, 2002/2003).

3.2 HISTORICAL BACKGROUND

TB has been a major cause of suffering and deaths since time immemorial. Thought to be one of the oldest human diseases, the history of TB is at least as old as the mankind. The word tuberculosis is a derivative of the Latin word "tubercula" which means "a small lump". J.L.Schonlein, Professor of Medicine at Zurich, is credited to have named the disease "tuberculosis" (Mohan and Sharma, 2001b). Although the infectious nature of TB was established by Villeman around 1865, the protean nature of the clinical manifestation delayed understanding of the disease until Koch's discovery of the causation of agent in March 24, 1882 (Jolik *et al*., 1992).

A look at the history of TB reveals that it took several thousands of years for humans to identify the causative organism, another 60 years to arrive at effective treatment (Maher *et al*., 1997). TB has recently re-emerged as a public health problem after its considerable decline during past decades. The 1990's have witnessed a resurgence of TB. Re-emergent TB is fuelled by the pandemic HIV and AIDS and by single and multi drug resistance (Tanuphasiri and Kladphuang, 2002). In April 1993, WHO declared TB as a global emergency (Sohn *et al*., 2003).

3.3 TRANSMISSION OF TUBERCULOSIS

TB is spread from person to person through the air by droplet nuclei, particles 1-5µm in diameter that contains *M. tuberculosis complex*. The risk of infection for persons who encounter *M. tuberculosis* is influenced by many factors concerning the source patient,

the environment and the individuals who come in contact with the pathogen (Harries *et al*., 1998; Ponticeellio *et al*., 2001). The AFB smear positive cases identifies the most infectious patients, however both theoretical consideration and empirical observation indicates transmission does occur from smear negative (SN) patients. 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 organisms and report does show that patient with SN culture positive TB appears to be responsible for about 17% of TB transmission (Behr *et al*., 1999).

3.4 SOCIO ECONONOMIC RISK FACTORS OF TUBERCULOSIS

TB doesn't respect anybody. It affects both rich and poor people, in both sexes and in all age groups. The threat of TB to mankind is no longer restricted to developing nations since it has now returned to industrialized countries due to AIDS epidemic and the emergence of Multidrug resistant (MDR) strains (Mehra and Rajalingam, 2001). Nearly two third of all cases are found in racial and ethnic minorities. Greater risks are prisons inmates, alcoholics, the elderly, intravenous drug users and foreign born persons from areas of the world with a high prevalence of the disease. The TB case rates are influenced by the race, sex and age of the population group (Jolik *et al*., 1992). TB is a social disease with medical implications. Poverty and TB have always been close allies and has been always occurred disproportionately among disadvantaged populations such as homeless, malnourished, and overcrowded (Dunlap *et al*., 2000). Several factors are responsible for the outbreak of TB in the region: the detoriation of socio – economic conditions, with low income and inadequate education, and great ignorance of the disease, as there are facts that support that patients often stop taking medicine when their symptoms have disappeared (Ulukanligil *et al*., 2000).

Humans are very susceptible to tuberculous infections but remarkably resistant to tuberculous disease. It has been estimated that for all persons newly infected with tubercle bacilli about 5% will have clinical disease within a year of their infection. The remainders carry a life long risk of potential disease. Host immunity response to *M. tuberculosis* seems to play a major role in determining the ultimate outcome of persons

who encounter the pathogen. It is estimated that the rate of TB patients with AIDS is 100 times than that in general population (Jolik *et al*., 1992).

3.5 DETERMINANTS OF PATHOGENECITY and PATHOGENESIS

A number of properties are usually associated with the capacity of virulent strains of *M. tuberculosis* to produce progressive disease. Cord factor and sulphatides are the recognized virulent factors of *M. tuberculosis* (Jolik *et al*., 1992).

M. tuberculosis produces neither exotoxins nor endotoxins. The tubercle bacillus owes its virulence to its ability to survive within the macrophage rather than production of a toxic substance. The immune response to the bacillus is of the cell-mediated type which, depending on the type of T helper cells involved, may either lead to protective immunity and resolution of disease or to tissue destroying hypersensitivity reactions and progress of the disease process (Greenwood *et al*., 1998).

In 90% of all cases, *M. tuberculosis* infection results in latency rather than active disease, with the pathogen being contained within granulomatous lesions at the site of primary infection. Failure of this containment leads to reactivation of post primary TB (Ulrichs *et al*., 2005).

Post-primary TB usually affects the lungs but can involve any part of the body. The characteristic features of post- primary PTB are the following: extensive lung destruction with cavitation, positive sputum smear, upper lobe involvement, without any intrathoracic lymphadenopathy (Harries *et al*., 1997).

The main component of the host defense against *M. tuberculosis* is a cell mediated immunity, which involves bidirectional interactions between T-Cells and cells of monocyte /macrophage lineage through specific cytokines. The main effector cell is the CD_4^+T-lymphocyte. Nevertheless, the phenotype of responding T cells is not fully defined, and other types of T cells, such as the CD8+ and T cells, have been recognized to contribute to protective immunity against *M. tuberculosis* (Ponticeellio *et al*., 2001).

3.6 ACID FAST BACILLI

M. tuberculosis is slender, straight or slightly curved rod with rounded ends. The organisms vary in width from 0.3-0.6 µm and length from 1-4 µm. The bacilli are non motile, non sporogenous and non-encapsulated. The most distinctive property of mycobacterial cell is its unusual cell wall, a multilayered structure that contains N-glycolylmuramic acid instead of N-acetylmuramic acid and very high lipid content. Because of this distinctive property within the genus mycobacterium, it has its characteristic staining (Jolik *et al*., 1992).

Mycobacteria are difficult to stain. The large amounts of lipids present in their cell walls makes them impermeable to the gram stain, and appearance of mycobacteria in a gram stained specimen may be variable. Mycobacteria are able to form stable complexes with certain aryl methane dyes such as fuchsin and auramine O. Although the exact nature of the acid-fast staining reaction is not completely understood, phenol in the primary stain allows penetration of the stain. The cell wall mycolic acid residues retain the primary stain even after exposure to acid-alcohol or strong mineral acids. A counter stain is employed to highlight the stained organisms for easier microscopic recognition. The acid fast nature of an organism can be determined by several methods. In carbol fuchsin staining procedures AFB appear red against a blue or green background (Collee *et al*., 1996). Fluorescent staining is regarded as a more reliable method due to more intensive binding of mycolic acids of the bacilli to phenol auramine, and so the bacilli stand out sharply against black background to allow rapid and accurate screening under low power objective (Ponticeellio *et al*., 2001).

3.6.1 SPUTUM SMEAR MICROSCOPY

TB remains a major public health problem in many parts of the world. Microscopic examination of respiratory specimens for AFB plays a key role in the initial diagnosis of TB, monitoring of treatment, and determination of eligibility for release from isolation (Peterson *et al*., 1999). Sputum smear microscopy is the mainstay of diagnosis of TB. It is efficient and can confirm the disease. Besides this, other advantages 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 its primarily role in case detection as it can detect the most infectious subset of patients (Selvakumar, 2003; Habeenzu *et al*., 1998; and Parekh and Kar, 2003). The visualization of AFB in sputum or other clinical materials should be considered only presumptive evidence of TB, because stain does not specifically identify *M. tuberculosis* (Forbes *et al*., 1998). It has been observed that between 10^4-10^5 tubercle bacilli per ml of sputum are required for direct microscopy. Sputum specimens from patients with cavitary diseases are most often sputum smear positive (Katoach, 2004; Parekh and Kar, 2003).

The microbiological diagnosis of PTB plays a key role in routine and Tuberculosis Control Programmes in developing countries. The cornerstone of the diagnosis of TB is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. A direct relationship was demonstrated between positivity in results and mucopurulent sputum. Positivity and bacillary count were found to be lower in thin smears (Perkins, 2000; Kuszniez *et al*., 2004).

However, direct microscopy of sputum, though rapid has low sensitivity (Habeenzu *et al*., 1998). In one study by Aber *et al*. (1980) found that in several African laboratories, the sensitivity of direct microscopy ranged from 8.8% to 46.4%. The sensitivity of direct sputum microscopy may be increased by concentration method. Concentration of AFB in clinical specimens is an important step in the laboratory diagnosis of mycobacterial diseases. Kocchar (2002) found that out of 1484 samples, 9.3 % positivity rate was observed on direct microscopy with the increase of yield by 30% after concentration.

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 classical 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. Petroff's

concentration method with sodium hydroxide was described in 1968 and is widely used as it increases the sensitivity of sputum smears and also that of cultures since the mycobacteria concentrated in the deposits are viable (Perkins, 2000; Garay, 2000). But when sputum is not adequate *i.e.* with high number of squamous epithelial cells, the sensitivity of the method is low. Many studies reported that concentration methods by the use of NaOCl solution increased the yield of the AFB by more than threefold compared with the direct microscopy of sputum ($P< 0.05$) (Gebre, 2003). However sediments after concentration with NaOCl cannot be used for culture of mycobacteria. Concentration method also facilitates the examination of the slides and reduces the time required for microscopy (Gebre *et al*., 1997).

Three types of staining procedures are commonly used in the laboratory for rapid detection and confirmation of AFB: Fluorochrome, ZN, and Kinyoun. While the specificity of microscopy is high, ranging between 98-99%, the sensitivity is relatively poor at 50-70% or even lower in the public health setup. The sensitivity of the acid-fast smear examination for the diagnosis of mycobacterial infection is lower than that of culture methods. The factors influencing sensitivity includes type of specimen, concentrations of mycobacteria in specimens, and staining techniques. Respiratory specimens yielded the highest smear positivity rate, followed by tissue specimens and cerebrospinal fluid. Furthermore, if more than one respiratory tract specimen is submitted to the laboratory, 96% of patients with PTB may be detected by the acid-fast smear examination (Kar *et al*., 2003; Balows *et al*., 1991).

Fluorochrome stain is more sensitive than the conventional carbol fuchsin stains because the fluorescent bacilli stand out brightly against the background; the smear can be initially examined at lower magnifications (250X-400X), and therefore more fields can be visualized in a short period. This makes the method appropriate for use in central or large laboratories with heavy workloads but is less feasible in small laboratories because of the associated cost, equipment maintenance and lower specificity (CDC, 2005). The classic carbol fuchsin (ZN) requires heating the slide for better penetration of stain into the mycobacterial cell wall hence it is also known as the hot stain procedure. Kinyoun acid-fast stain is similar to ZN but without heat; hence the term cold stain. Kinyoun

requires a high concentration of basic fuchsin and phenol or the addition of a detergent thereby avoiding the need for heat. In this study, a new, cheaper, safer and easier staining method named as Modified cold stain is described for the demonstration of AFB (Forbes *et al*., 1998; Tanuphasiri and Kladphuang, 2002). Though this technique is not popular but it is an improved acid fast staining technique, which is far easier, safer rapid and invasive technique. The technique was modified and simplified by eliminating heating step and combining the stages of decolourisation and counter staining. When acid-fast organisms are observed on a smear, results must be quantified to be meaningful because this quantitation estimates the number of bacilli being excreted; the extent of a patient's infectiousness can be assessed for clinical and epidemiological purposes (Forbes *et al*., 1998).

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

Singh and Parija (1998) compared the light microscopy of ZN with that of fluorochrome dye for detection of AFB and reported that out of 2600 clinically suspected patients, sputum specimens from 1,104 patients were found to be positive for AFB. These included sputum from 975 (37.5%) patients positive for AFB both ZN and auramine staining methods and sputum from additional 129 (4.96%) patients positive for AFB by auramine staining only.

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

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

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

specimens with positive cultures and negative smear yielded less than 25 colonies, whereas there were greater than 25 colonies from 93% of the specimens with positive smears and cultures.

In the study done by Githui *et al*. (1993), a total of 1480 sputum specimens collected from patients with suspected PTB were analyzed. Culture results were used as the gold standard for assessment. Specificity was 97% and 96% for FM and ZN methods, respectively. The sensitivity of the FM method was 80% and that of the ZN method 65% ($p < 0.001$). Overall agreement was 86.8%. The use of FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears. The method is economical in both time and expense and is recommended for laboratories handling large numbers of sputum specimens.

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

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

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

by both methods. In the remaining five samples, 2 were positive by only ZN staining and 3 by only cold staining. Kocchar (2002) also observed that the simple MC staining and simplified concentration are valuable alternatives for mycobacteria in busy clinical laboratories.

In a study Somoskovi *et al*. (2001) evaluated the routine performance of four different staining methods (Kinyoun, ZN, auramine, AR) and it was found that Kinyoun cold carbol fuchsin method is inferior to both the ZN and Fluorochrome (auramine and/or AR) methods. A comparison of readings with the four different staining methods revealed a significantly higher sensitivity of AR Vs Kinyoun ($p < 0.01$), ZN ($p = 0.01$), and auramine ($p < 0.02$). The difference between auramine and Kinyoun ($p = 0.064$), and auramine and ZN ($p = 0.089$) which was close to being significant, while there was no significant difference between Kinyoun and ZN ($p = 0.73$).

Selvakumar *et al*. (2002) evaluated a two step cold staining methods for detecting AFB in sputum smears which has been developed in Tulip Diagnostics (Goa, India) with that of ZN in total of 244 sputum samples and has found that the concordance between the methods was 90% (kappa value=0.7). The performance of cold method and ZN method was similar when their smear results were compared with culture results (cold method Vs culture, kappa=0.61; ZN method Vs culture, kappa=0.67).

Deshmukh *et al*. (1996) when made comparative study of ZN, cold stain and modified Schaeffer and Fulton method in sputum examination for AFB, they reported that out of 187 sputum samples studied, 67(35.82%) were reported positive by ZN stain and cold stain method while 65(35.29%) were reported positive by Schaeffer and Fulton method.

Much study reveals that the ability of any screening test to correctly identify diseased patients is directly related to the prevalence of the disease in question. The continuing use of smears for the detection of acid-fast bacilli when the prevalence of TB is declining will produce increasing numbers of false-positive (FP) results. Data suggest that the smear is a poor screening technique in a population where the prevalence of TB is low (Boyd and Marr, 1975). But Gordin and Slutkin (1990) reported that the positive predictive value (PPV) of acid-fast microscopy was 97.9% and 100% in both low and high laboratory prevalence periods of *M. tuberculosis.*

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

3.6.2 LIMITATIONS OF SPUTUM SMEAR MICROSCOPY

Although relatively rapid, simple, and inexpensive, sputum microscopy suffers from a major drawback of low sensitivity. This disadvantage of the method was already apparent before the advent of HIV, since the sensitivity range of 30%-40% with single sputum specimen and 65-70% with repeated smear examinations (Bruchfeld *et al.*, 2000). The immediate diagnosis of TB by direct sputum examination ranges from 40-75%. Isolation of the organism by culture and subsequent identification by biochemical test is time- consuming; it takes about 4-6 weeks and has only 40-60% sensitivity (Sohn *et al.*, 2003).

Many variables influence the results of microscopy which include 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).

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

It is recommended that a negative result should only be reported following the examination of at least 100 (in low-income countries) and preferably 300 (in

industrialized countries) microscopic immersion view fields (or equivalent fluorescent view fields). Therefore, when microscopy is performed correctly, it can be time-consuming and laborious (Somoskövi *et al.***,** 2001).

Negative results and slow reporting ultimately may erode patient faith in the services of the laboratory and of the health system. And again, a negative smear result does not exclude the diagnosis of tuberculosis, as about 55% of PTB cases worldwide, harbor lower bacillary load, so that the sputum is negative upon microscopic observation (Parekh and Kar, 2003).

Further the use of sputum smear as a screening procedure for the presumptive diagnosis of PTB has recently been criticized following the finding by several large laboratories that up to 55% of specimens with positive smear failed to grow in culture while 30% are smear negative but culture positive (Barez *et al.,* 1995). Many data suggest that the sputum smear microscopy is a poor screening technique in a population where the prevalence of TB is low and where their is increasing burden of HIV and AIDS (Prasanthi and Kumari, 2005; Boyd and Marr, 1975).

3.7 DIAGNOSTIC APPROCH FOR TUBERCULOSIS

Rapid and accurate diagnosis of symptomatic patient is a cornerstone of global TB control strategies. For the success of TB control, the transmission of tubercle bacilli in a community should cut either infection, onset of TB or the progress of TB. Prevention of TB infection could be achieved by the early detection of TB cases and their cure by the treatment (Perkins, 2000; Shimao, 2005).

Ideal TB diagnosis should address the four focal aspects of TB control in low income countries, i.e.

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

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

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

IV. 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 on a country wide basis and on a long- term (Kar *et al*., 2003).

Microbiology laboratory contributes to the diagnosis and management of TB in:

I. Detection and isolation of mycobacteria

II. Identification of species of the isolate

III. Antibiotic susceptibility testing of the isolate (Rattan, 2001).

The global strategy for controlling TB is through a six month treatment with anti-tuberculous drugs. The use of chemotherapy renders patients non infectious and the chain of patient to patient transmission is broken. Fundamental to this process is the correct diagnosis of patients. The problem of diagnosing TB is that no symptom or sign is typical to it, neither presence of bacilli is indicative of disease; TB is still primarily diagnosed using microscopy for detection of AFB. It remains the cornerstone for laboratory diagnosis of TB in clinical practice as well as the National tuberculosis control programs of the large majority of under –developed countries.

AFB microscopy has been characterized as a simple, inexpensive and rapid technique, which has high specificity for mycobacteria, but AFB microscopy has the major limitation of its low sensitivity. A worldwide effect is under way to develop new tools to diagnose TB, spearheaded by the WHO under its tuberculosis diagnostic initiative (TBDI) (Kar *et al*., 2003).

Advances in techniques for the diagnosis of TB are also being made in an attempt to address this problem. These advances include radiometric cultures, detection of tuberculostearic acid (gas chromatography-mass spectrometry) and mycobacterial

antigens (enzyme-linked immunosorbent assays), DNA probes, and nucleic acid amplification (NAA) system such as PCR (Wright *et al*., 1998).

Remarkable progress has recently been made upgrading the speed and quality of mycobacteriology diagnosis services in developed countries, but for most of the world where TB is a large public health burden those gains are still unrealized (Perkins, 2000).

The use of stained sputum smear microscopy for detection of AFB, however still remains the most available, easy to perform, inexpensive and rapid diagnostic test for TB and this is especially true for laboratories in developing countries, where limited resources and financial constraint often do not allow even culture isolation as a diagnostic option (Rattan, 2001).

Four diagnostics are routinely used for diagnosing TB in developing countries. These are direct visualization of bacilli by either microscopy or culture on specific media, or indirect methods such as radiography and tuberculin testing. Of these four diagnostics, the specificity of microscopy is about 99%, but its sensitivity ranges between 50-70% and less in public health set up (Kar *et al*., 2003).

The inoculation of concentrated bacilli from processed clinical specimens on solid media is a standard approach for confirmation of TB. Culture methods are more sensitive and specific than microscopy for detection of bacilli, since approximately 10-100 mycobacteria per milliliter of sample is required for positive result while approximately 10^4organism per ml of sputum is required to be seen by microscopic examination. Despite its enhanced sensitivity as well as specificity, culture is of little clinical use, because necessarily lengthy time required for isolation of mycobacteria poses a special problem for the laboratory. Moreover culture requires at least a moderately well – equipped laboratory, which is not commonly available in many low income countries (Kar *et al*., 2003; Gebre *et al*., 1997).

Tuberculin test is widely used for the diagnosis of TB, especially in children, as well as for epidemiological investigations of infection (Kar *et al*., 2003) However, in populations

where there is high prevalence of TB, the tuberculin skin test is of little clinical value in the diagnosis of TB, mainly in adults because a positive tuberculin skin test does not by itself distinguish *M. tuberculosis* infection from tuberculosis disease (Harries *et al*., 1997).

Diagnosis of TB by radiological means is also important means of case detection in clinical practice as well as the National tuberculosis control programs among large majority of under–developed countries; however sensitivity of chest X-ray for early diagnosis of recurrent PTB is not very high, and also the low specificity of chest X ray, if used for the diagnosis of smear–negative TB, it may risk with high levels of over diagnosis. Hence, bacteriological examinations of sputum are considered more important irrespective of chest X-ray finding for more accurate diagnosis of TB (Kivihya, 2004; Ito, 2005).

The limitation of these routinely used tests and the need to speed treatment in light of the impending HIV epidemic, have forced and encouraged to improve diagnostic tools. In recent years, much hope has been laid on the development of molecular techniques in the routine tuberculosis laboratory, among which four techniques that are increasingly used in clinical laboratories: PCR to detect mycobacterial DNA in clinical specimens, nucleic acid probes to identify culture, restriction fragment length polymorphism analysis to compare strains for epidemiologic purposes and genetic-base susceptibility testing methods for rapid detection of drug resistance (Su, 2002).

4.8.1 CULTURE MEDIA

Solid media

Solid media may be egg based or agar based. This media contains malachite green, a dye that suppresses the growth of contaminating bacteria. Of the egg based media, LJ medium is most commonly used in clinical laboratories. In general, LJ medium recovers *M. tuberculosis* well but is not reliable for the recovery of other species. Petragnani medium contains about twice as much malachite green as does LJ medium and is most

commonly used for the recovery of mycobacteria from heavily contaminated specimens. Agar based media are transparent and provide a ready means of detecting early growth of microscopic colonies. Colonies may be observed in 10-12 days in contrast to 18 to 24 days with opaque egg based media. The distinctive colony characteristic of *M. tuberculosis* is rough, tough, and buff colony (Collee *et al*., 1996; Rattan, 2001).

Liquid media

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

3.8.2 BACTEC AFB STSTEM

A radiometric detection system for mycobacterial growth developed by Middlebrook et al in 1977 has evolved into the BACTEC AFB system (Becton Dickinson diagnostic instruments, sparks, Md). Two types of BACTEC mycobacterial culture media are currently available. BACTEC 12B medium is a broth medium that has been reported to yield more positive cultures from clinical specimens than other media. BACTEC 13A is used for blood and bone marrow specimens (Rattan, 2001).

3.8.3 SEROLOGY

Use of serum for the detection of different antigens of *M. tuberculosis* can be done by different ELISA techniques. Detection of PPD and lipoarabinomannon antigens of *M. tuberculosis* can also be done (Pokhrel, 2004). There have recently been significant improvements in serologic tests and in our understanding of the humeral immune response, but the HIV epidemics and its impact on immunity have threatened its progress (Perkins, 2000). Serological techniques are not useful in control programmes due to the lack of sensitivity and specificity (Gebre *et al*., 1997).

3.8.4 NUCLEIC ACID AMPLIFICATION

Among the new approaches for the rapid diagnosis of TB, the NAA methods are the most promising, but the technology is not applicable to control programmes in developing countries (Gebre *et al*., 1997). One of the most important technical advances for TB laboratories in industrialized countries has been the development of NAA assays, two of which the Enhanced *M. tuberculosis* Direct Test (Gen-Probe) (Gen-Probe MTD) (San Diego, CA) and the Amplicor *M. tuberculosis* Test (Roche Diagnostic Systems) have been approved by the US Food and Drug Administration (FDA) for the detection of *M. tuberculosis* in respiratory specimens that are smear-positive for AFB and which have been increasingly used in industrialized settings (Hanna and James, 2000; Ito, 2000). From the data reviewed by the FDA, the specificity (100%) and sensitivity (95%/96% in the two studies) of these two tests in AFB smear-positive specimens were found to be comparable to the Accuprobe (Gen-Probe) for identification of *M. tuberculosis complex* in culture, with the advantage that the direct amplification test (DAT) results are available much sooner. The DAT are significantly more sensitive than the AFB smear. PCR is not only simple and fast, but also very sensitive and specific to amplify even a singe molecule of DNA. Even if, PCR tests are expensive currently, they will help in the overall savings to the patient by providing a timely diagnosis (Sohn *et al*., 2003). However, in AFB SN samples, the specificity, sensitivity, and positive predictive value were 96/99%, 48/53% and 24/58%, respectively, in the two studies. Usefulness of commercial NAA Test kits to diagnosis SN-PTB is limited in the point of sensitivity. The commercial versions of NAA tests tend to be simpler and also more reliable , but because of the technical support and quality control required by all these tests, and its high cost makes them unsuitable for most developing countries (Ito, 2000; American thoracic society, 1997).

PCR-based sequencing has been commonly used to identify many mycobacterial species. DNA probes have been widely used for species determination of the most commonly encountered mycobacteria. High-density oligonucleotide arrays (DNA microarrays) also have been applied to simultaneous species identification and detection of mutations that confer rifampin resistance in mycobacteria (Kivihya *et al*., 2004). Condos *et al*. (1996)

reported overall sensitivity and specificity of the PCR assay for a diagnosis of TB was 95% and 89%, respectively. The PCR assay correctly identified 39 of 41 patients with proven PTB while 26 (63%) of whom were sputum-smear negative.

PCR can be considered as an alternative to ZN staining in combination with chest X-ray for diagnosis of TB since it has shown major improvement over standard techniques, however there is currently insufficient information on their clinical and public health utility. It is recommended that the currently available DAT should always be performed in conjunction with microscopy and culture, and each test result must be interpreted within the overall clinical setting in which it is used ((Kivihya *et al*., 2004; Hanna and James, 2000).

Hence, 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 (Jain *et al*., 2002). No other diagnostic tool offers the affordability as well as efficiency in diagnosis of TB in public health setup, as sputum microscopy does.

CHAPTER – IV

4. MATERIALS AND METHODS

4.1 MATERIALS AND CHEMICALS USED

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

4.2 METHODOLOGY

4.2.1 SETTINGS

This study was conducted in Tuberculosis Research Laboratory at BPKIHS, Dharan, Nepal from May 1st, 2005- Nov 15th 2005. The PTB patients, who visited this hospital, were from Eastern Region of Nepal. The ethical clearance was taken from the BPKIHS ethical committee as per the institute guidelines.

4.2.2 TYPES OF STUDY: Cross-sectional study

4.2.3 INCLUSION CRITERIA

Routine fresh clinically suspected PTB patients referred from out patient Department (OPD) to Tuberculosis Research Laboratory were included.

4.2.4 EXCLUSION CRITERIA

Patients suspected of PTB from In- Patient Departments like: medical wards, tropical wards, patient with EPTB and patients undergoing Anti tuberculosis treatment (ATT) were not selected.

4.2.5 SAMPLE SIZE

From the pilot study done in Tuberculosis Research Laboratory, BPKIHS, the positive rate found among OPD new suspected patients excluding ward patients, tuberculosis clinic patients, and EPTB patients was approximate 17%. In the present study maximum avoidable error was taken 20% and then the sample size was calculated. The sample size in the study is 500 clinically suspected PTB patients.

4.2.6 SAMPLE COLLECTION

Three sputum samples from each patient, one "on spot" and two early morning samples were collected on the consecutive days. The patients were provided with sterile, leak proof, disposable and appropriately labeled wide mouthed container. The patients were instructed to provide about 5ml deep- cough sputum, not saliva or nasal secretions.

4.2.7 STUDY METHODS

A Semi structured proforma was designed for clinical evaluation. (Please refer to Appendix No 2). For staining and culture, sputum samples were first decontaminated and concentrated by centrifugation using 4% NaOH, according to modified Petroff's method. Smears on four individual slides were prepared for four different staining methods i.e. ZN, Kinyoun, MC stain, and Fluorochrome stains. Remaining sediments were inoculated in the Lowenstein-Jension (LJ) media.

With culture employed as the gold standard for diagnosis, it was compared with the respective staining techniques.

In recording and reporting of microscopic results, the following reporting scale was used for ZN stain, Kinyoun stain and MC stain as per the guidelines given by IUATLD (Kantor *et al*., 1998).

NUMBER OF BACILLI SEEN IN A SMEAR	RESULTS REPORTED
No AFB per 300 oil immersion fields	*negative*
1-9 AFB per 100 oil immersion fields	*record the exact number*
10-99 AFB per 100 oil immersion fields	*1+*
1-10 AFB per oil immersion field	*2+*
>10 AFB per oil immersion field	*3+*

The number of AFB found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease.

In order to equilibrate the number of bacilli observed with FM (400x magnification) to the number of bacilli observed with immersion microscopy (1000x magnification), the values were divided by 4 (Kantor *et al*., 1998).

4.2.7.1 TREATMENT OF SPUTUM

Decontamination and centrifugation by **modified Petroff's method**

PROCEDURE

1. Whole specimen was transferred into 15 ml plastic centrifuge tube.
2. Equal amount of 4%NaOH was added, mixed vigorously and was let to stand for 15 minutes.
3. The specimen was centrifuged at 3000 rpm for 15 minutes.
4. The supernatant was discarded and distilled water was added and again centrifuged at 3000 rpm for 15 minutes.
5. Supernatant was decanted. The resuspended sediment was inoculated to LJ media and was used to prepare four slides respectively for four different staining.

4.2.7.1.1 INOCULATION AND INCUBATION PROCEDURES

Single slope slant per specimen were inoculated each with one 4 mm loopful of the centrifuged sediment, distributed over the surface of LJ media. All culture tubes were incubated at 37^0 C until growth was observed. Those tubes in which growth was not observed after 8 weeks were regarded as negative and were discarded.

4.2.7.1.2 CULTURE EXAMINATION SCHEDULE

All cultures were examined after 48-72 hours of inoculation to detect any contaminants. Thereafter cultures were examined on 7th day for rapid growers once weekly thereafter, up to 8 weeks, for slow growers after which a definitive result was obtained.

4.2.7.1.3 OBSERVATION OF CULTURES

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). Growth of mycobacteria was confirmed by typical colony morphology and microscopy for AFB. Cultures that showed no growth after 8 weeks were regarded as “negative” and were discarded.

4.2.7.1.2 PREPARATION OF SMEAR

1. Concentrated sputum sample was smeared evenly with an uneven end of broom stick on the four slides each labeled for staining techniques respectively, the smear size being 2 cm x 3 cm and it was not too thick.
2. The smear was air dried before being fixed.
3. The smear was methanol fixed.
4. The slides were then placed in serial order on the staining rack with the smeared slides facing upward ensuring slides do not touch each other.

STAINING METHODS

I. ZIEHL-NEELSEN STAIN

1. 1% carbol fuchsin was poured to cover the entire surface of the slides.
2. The slides were heated underneath until vapour start rising.
3. The slides were allowed to stand for 5 minutes.
4. The slides were then rinsed with tap water and excess water was drained off.
5. The slides were decolourised with 3% acid alcohol for 3 minutes.
6. The slides were rinsed thoroughly with tap water and excess water was drained off.
7. The slides were flooded with 0.3 % malachite green solution and were let to stand for 1 minute.
8. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry.
9. The slides were examined under microscope in1000x oil immersion.

II. KINYOUN STAIN

1. Concentrated carbol fuchsin was poured to cover the entire surface of the slides.
2. The slides were allowed to stand for 5 minutes.
3. The slides were then rinsed with tap water and excess water was drained off.
4. The slides were decolourised with 3% acid alcohol for 3 minutes.
5. The slides were rinsed thoroughly with tap water and excess water was drained off.
6. The slides were flooded with 0.3 % malachite green solution and were let to stand for 1 minute.
7. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry.
8. The slides were examined under microscope in1000x oil immersion.

III. MODIFIED COLD STAIN

1. Concentrated carbol fuchsin was poured to cover the entire surface of the slides.
2. The slides were allowed to stand for 10 minutes.
3. The slides were then rinsed with tap water and excess water was drained off.
4. The slides were decolourised and counter stained at same time with Gabbet methylene blue for 2 minutes.
5. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry.
6. The slides were examined under microscope in1000x oil immersion.

IV. FLUOROCHROME STAINING

1. Entire smear was flooded with auramine O and was allowed to stain for 15 minutes, ensuring that staining solution remains on smears.
2. The slides were rinsed with distilled water and excess water was drained off.
3. The slides were decolourised with 1% acid alcohol for 2 minutes.
4. The slides were rinsed with distilled water and drained off.
5. The slides were flooded with potassium permanganate and allowed to counter stain for 2 minutes.
6. The slides were rinsed with distilled water and drained off.
7. The slides were allowed to air dry.
8. The slides were examined under fluorescence microscope at low power as soon as possible after staining.

4.2.7.3 REPORTING OF TB CASES

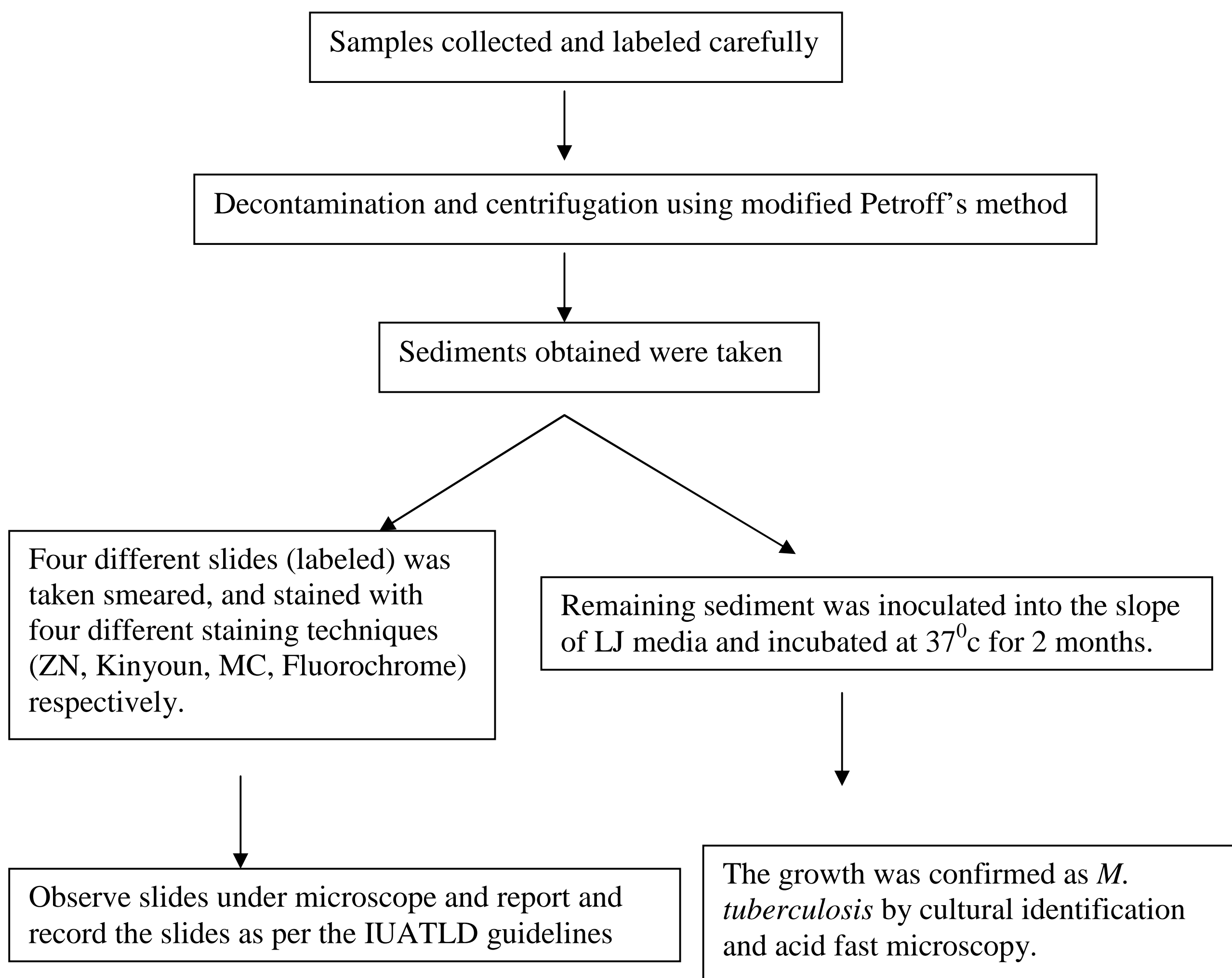
A patient was considered as a “TB-positive subject” if one of the three sputum specimens had a positive culture and as a “non-TB subject” if none of the three sputum specimens showed growth.

4.3 STATISTICAL ANALYSIS

The sensitivity, specificity, positive predictive value and negative predictive value were calculated using statistical product and service solution (SPSS) software version 11.5.

4.4 EXPERIMENTAL DESIGN

The experiment was designed to evaluate different staining techniques for detection of AFB in primary diagnosis of PTB.



Flow chart: Experimental design for sample processing and analysis

Sources: Ulukanligil *et al*. (2000)

CHAPTER – V

5. RESULT

During the study period, a total of 1365 specimens out of 500 clinically suspected patients were analyzed and 109 (21.8%) of the 500 patients were diagnosed as having PTB by the isolation of the organism from culture of sputum. 1.2% of PTB suspects were identified positive with all four staining methods but were not verified by culture.

In the study group, Table 1 shows age and sex wise distribution of PTB suspects in the study group. In 500 PTB suspects, 59.6% (n=298) were males and 40.4% (n=202) were females, among whom 24.2% (n=73) male and 17.8% (n=36) female were diagnosed as PTB cases. Maximum numbers of TB cases were observed in the economically most productive age group of 15-24 years (26.1%). TB was not diagnosed in suspected cases below 15 years. On the basis of other age wise distribution, no significant difference was seen in TB cases.

Table 1: Age wise and Sex wise distribution of TB cases in the study group

Age and gender		Number (n)	Tuberculosis	
			Positive %(n)	Negative %(n)
Age	Less than 15 years	27	0 (0)	100(27)
	15-24	111	26.1(29)	73.9(82)
	25-34	87	16.1(14)	83.9(73)
	35-44	75	22.9(18)	77.3(57)
	45-54	63	23.8(15)	76.2(48)
	55-64	71	25.4(18)	74.6(53)
	More than 64 years	66	22.7(15)	77.3(51)
Sex	Male	298	24.2(73)	75.8(225)
	female	202	17.8(36)	82.2(166)
Total		500	21.8(109)	78.2(391)

Considering the distribution of TB among genders, more males have been found to be infected than females, in all age groups (figure 1).

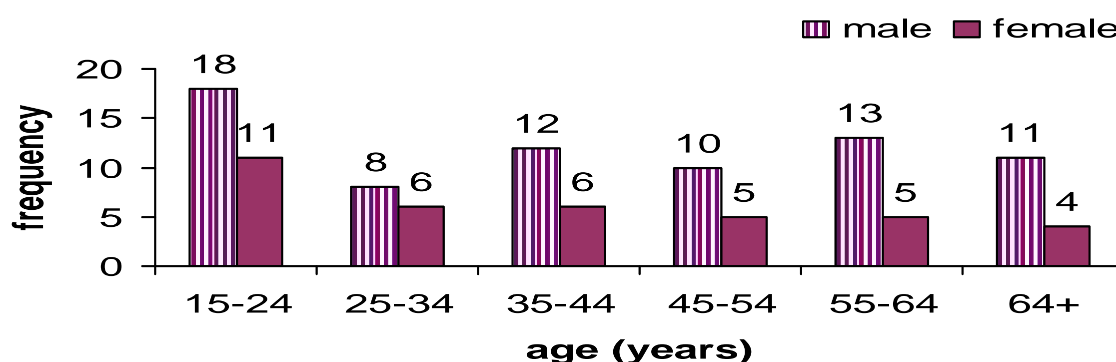


Figure1: Bar diagram depicting age wise sex distribution in culture positive cases

The present study evaluated the different staining techniques used in the primary diagnosis of PTB. The different staining techniques were ZN stain, Kinyoun stain, MC stain, and Fluorochrome stain methods. The staining methods were evaluated against culture on LJ medium, employed as ‘gold standard’.

The validities of the four staining methods using culture as the gold standard for TB diagnosis are shown in Table 2, Table 3, Table 4, and Table 5. Table 2 shows that the total positive results obtain by ZN were 71 (14.2%) and out of which the true positive (TP) was found to be 63 and false positive (FP) was found 8 in number (2%). ZN could diagnose 429 (85.8%) as total negative, out of which 383 of suspected cases were true negative (TN) and 46 (42.2%) of them were false negative (FN). The sensitivity of ZN was found to be 57.8% and that of specificity was found to be 98%. Positive predictive values (PPV) and negative predictive values (NPV) were found to be 88.7% and 89.3% respectively.

Table 2: Evaluation of the Ziehl-Neelsen stain and Culture results for the primary diagnosis of pulmonary tuberculosis

Staining methods	**Culture result**			**Sensitivity %**	**Specificity %**	**Predictive value**	
	Positive	Negative	Total			Positive %	Negative %
Ziehl-Neelsen							
Positive	63	8	71	**57.8**	**98.0**	**88.7**	**89.3**
Negative	46	383	429				
Total	109	391	500				

Table 3 shows that the total positive results obtain by Kinyoun were 69 (13.8%) and out of which the TP was found to be 61 and FP was found 8 in number (2%). Kinyoun could diagnose 431 (86.2%) as total negative, out of which 383 of suspected cases were TN and 48 (44.0%) of them were FN. The sensitivity of Kinyoun method was found to be 56.0% and that of specificity was found to be 98%. The PPV and NPV were found to be 88.4% and 88.9% respectively.

Table 3: Evaluation of the Kinyoun stain and Culture results for the primary diagnosis of pulmonary tuberculosis

Staining methods	**Culture result**			**Sensitivity %**	**Specificity %**	**Predictive value**	
	Positive	Negative	Total			Positive %	Negative %
Kinyoun							
Positive	61	8	69				
Negative	48	383	431	**56.0**	**98.0**	**88.4**	**88.9**
Total	109	391	500				

Table 4 shows that the total positive results obtain by MC were 72(14.4%) and out of which the TP was found to be 65 and FP was found 7 in number (1.8%). MC could diagnose 428 (85.6%) as total negative, out of which 384 of suspected cases were TN and 44 (40.4%) were FN. The sensitivity of MC was found to be 59.6% and that of specificity was found to be 98.2%. The PPV and NPV values were found to be 90.3% and 89.7% respectively.

Table 4: Evaluation of the Modified cold stain and Culture results for the primary diagnosis of pulmonary tuberculosis

Staining methods	Culture result			Sensitivity %	Specificity %	Predictive value	
	Positive	Negative	Total			Positive %	Negative %
Modified cold							
Positive	65	7	72				
Negative	44	384	428	**59.6**	**98.2**	**90.3**	**89.7**
Total	109	391	500				

Table 5 shows that the total positive results obtain by Fluorochrome method were 86(17.2%) and out of which the TP was found to be 78 and FP was found 8 in number (2%). Fluorochrome method could diagnose 414 (82.8%) as total negative, out of which 383 of suspected cases were TN and 31 (28.4%) of them could not be diagnosed by this method *i.e.* FN. The sensitivity of Fluorochrome method was found to be 71.6% and that of specificity was found to be 98%. The PPV and NPV were found to be 90.7% and 92.5% respectively.

Table 5: Evaluation of the Fluorochrome stain and Culture results for the primary diagnosis of pulmonary tuberculosis

Staining methods	Culture result			Sensitivity %	Specificity %	Predictive value	
	Positive	Negative	Total			Positive %	Negative %
Fluorochrome							
Positive	78	8	86				
Negative	31	383	414	**71.6**	**98**	**90.7**	**92.5**
Total	109	391	500				

Figure 2 shows the FP results produced by ZN, Kinyoun, MC stain and Fluorochrome stain were 2%, 2%, 1.8% and 2% respectively.

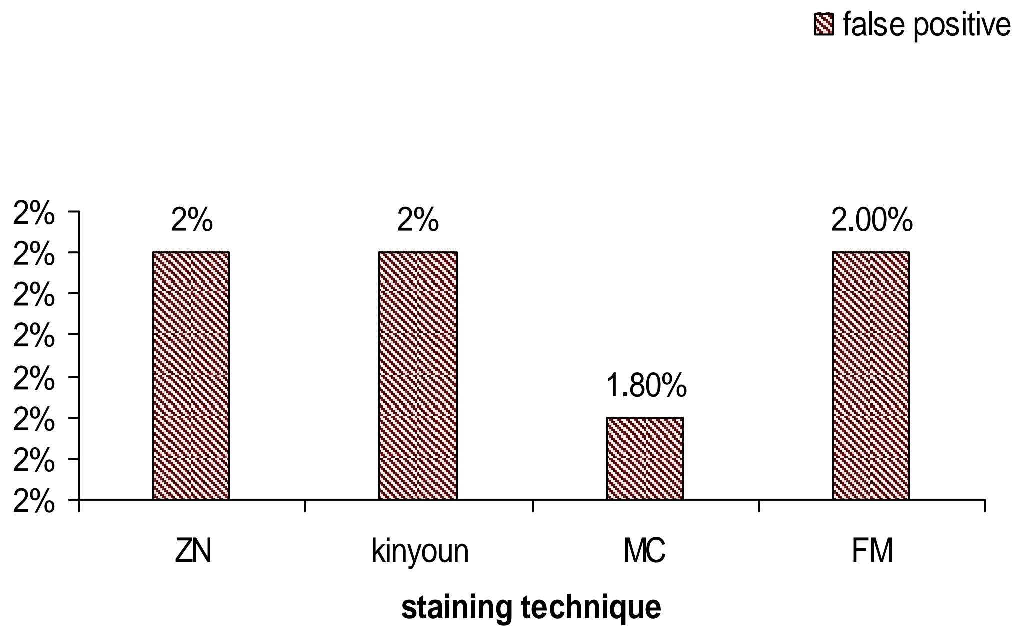


Figure 2: False positive result obtained in different staining techniques

Figure 3 shows that ZN, Kinyoun, MC and Fluorochrome staining methods gave 42.2%, 44%, 40.4%, and 28.4% FN rate results respectively.

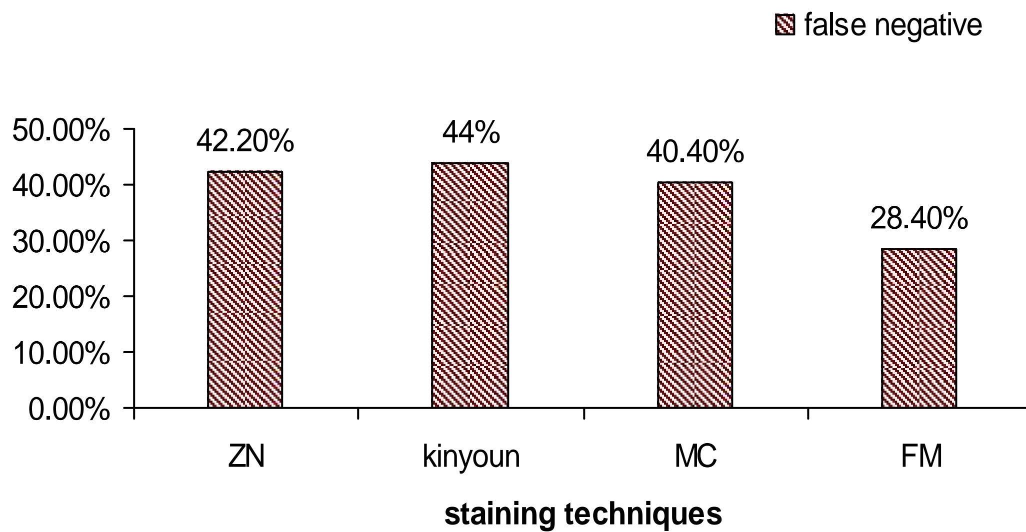


Figure 3: False negative result obtained in different staining techniques

Figure 4 shows the TP cases obtained by these different staining techniques and that was highest in Fluorochrome method (78 out of 86) and lowest in Kinyoun method (61/69).

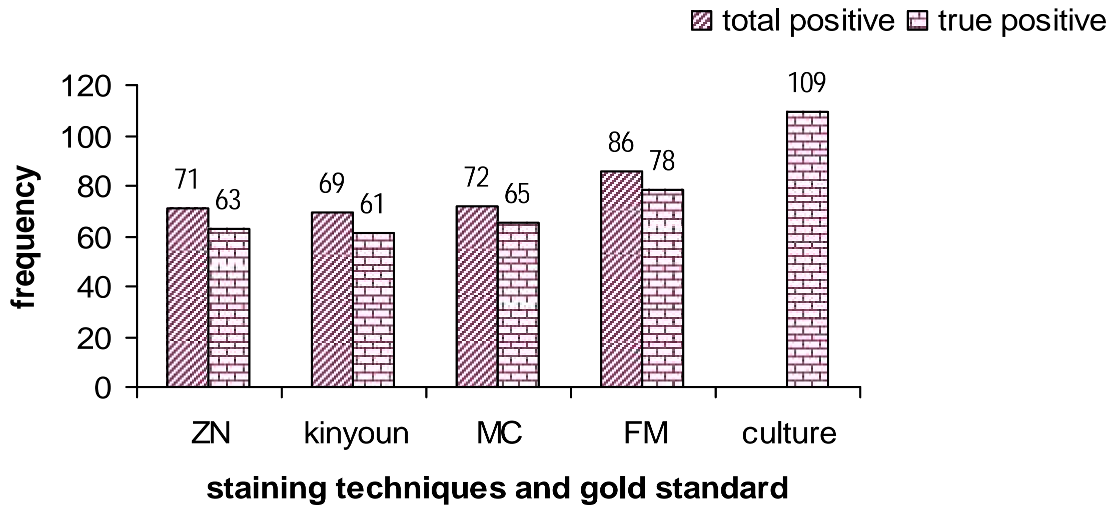


Figure 4: True positive obtained in different staining techniques with reference to culture

Figure 5 showed the percentage of suspected cases that were not diagnosed by all four staining techniques, and among them maximum cases were missed by Kinyoun method (36.69%).

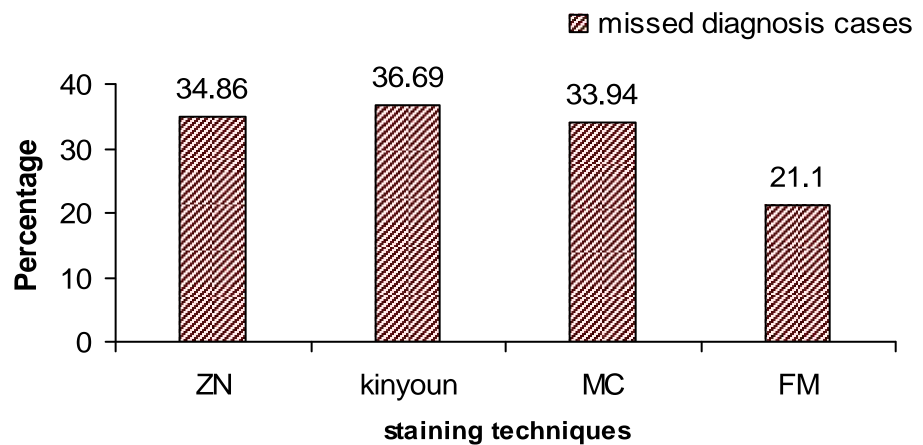


Figure 5: Percentage of missed diagnosis cases in different staining techniques

Figure 6 shows the total positive yield diagnosed by the four staining techniques in reference to the culture as gold standard. Fluorochrome staining (17.2%) gave the positive yield which is almost similar to that of the culture (21.8%). MC showed 14.4%, ZN showed 14.2% and Kinyoun staining technique showed 13.8%, which was lowest among the four staining techniques.

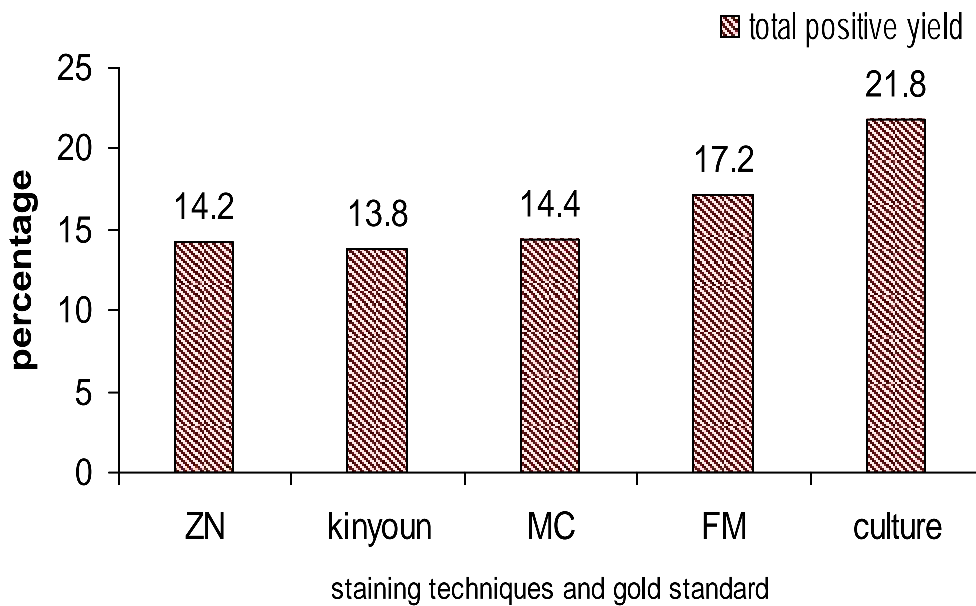


Figure 6: Total positive yield obtained by different staining techniques with reference to culture

Correlation of Kinyoun, Modified cold, Fluorochrome staining with Ziehl Neelsen staining method

In this present study, 500 sputum samples were examined in parallel. Table 6 shows the correlation of Kinyoun stain with ZN stain. The total yield of positive results was slightly higher by ZN: 71 (14.2%) positive compared with 69 (13.8%) positive by the Kinyoun stain. The positive agreement in between both stains was 91.5% and ZN could detect 8.5% more than Kinyoun method.

Table 6: Correlation between the Kinyoun and the Ziehl Neelsen staining techniques in slide reading of AFB- smear positive and negative

Kinyoun staining % (n)	Ziehl-Neelsen staining % (n)		Total % (n)
	Positive	Negative	
Positive	91.5 (65)	0.9 (4)	13.8 (69)
Negative	8.5 (6)	99.1 (425)	86.2 (431)
Total	100 (71)	100 (429)	100 (500)

Table 7 shows the correlation of MC staining method and ZN staining method. The total yield of positive results was slightly higher by MC staining method: 72 (14.4%) positive as opposed to 71 positive by ZN (14.2%). Positive agreement in between both stains was 98.6%. No difference was found in between these three staining techniques (figure 6).

Table 7: Correlation between the Modified cold and the Ziehl Neelsen staining techniques in slide reading of AFB- smear positive and negative

Modified cold % (n)	Ziehl Neelsen % (n)		Total % (n)
	Positive	Negative	
Positive	98.6 (70)	0.5 (2)	14.4 (72)
Negative	1.4 (1)	99.5 (427)	85.6 (428)
Total	100 (71)	100 (429)	100 (500)

Table 8 shows the correlation in between the Fluorochrome staining and ZN methods. Positive agreement in between the both stains was 98.6%. 3.7% (16) cases, which were not detected by ZN, were detected by Fluorochrome staining methods. The total yield of positive result by Fluorochrome was 86 (17.2%) as compared to 71 (14.2%) by ZN method. The positive yield is much greater in Fluorescent method than other staining methods (figure 6).

Table 8: Correlation between the Fluorochrome and the Ziehl Neelsen staining techniques in slide reading of AFB- smear positive and negative

Fluorochrome % (n)	**Ziehl- Neelsen % (n)**		Total **% (n)**
	Positive	Negative	
Positive	98.6 (70)	3.7 (16)	17.2 (86)
Negative	1.4 (1)	96.3 (413)	82.8 (414)
Total	100 (71)	100 (429)	100 (500)

Graphs below showed the relationship between sensitivity and specificity for respective staining techniques. In this Receiver Operator Characteristic curve (ROC), confidence interval was 95%.

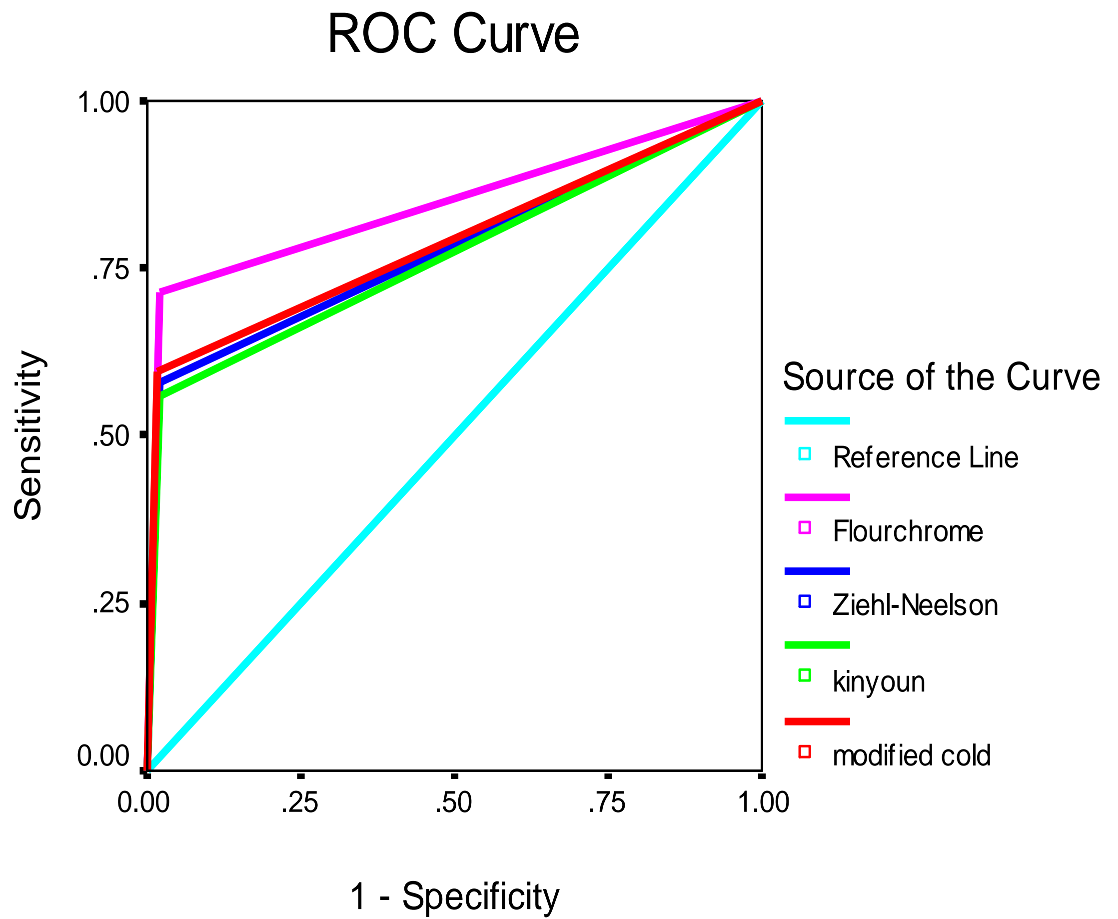


Figure 7: ROC curve in different staining techniques

CHAPTER - VI

6. DISCUSSION

According to the WHO guidelines for TB control, patient with more than three weeks history of cough should be screened for PTB with smear microscopy for AFB. Because the clinical signs and symptoms of PTB are not specific, the accurate performance of acid-fast microscopy is vital for the early detection of PTB patients for the adequate treatment, respiratory isolation, and contact investigation. Although acid-fast microscopy is more than 100 years old, it still remains the initial and most rapid step in the diagnosis of TB. Acid-fast microscopy is simple to perform and therefore could be applied successfully at any laboratory (Somoskovi *et al*., 2001). The added advantage of sputum smear microscopy is that it has very close relation with infectiousness: patient who are sputum smear positive and culture positive are far more likely to be infectious than culture positive but smear negative (Narain *et al*., 1971).

The usual staining laboratory technique for staining AFB used worldwide has been the ZN method, which has also been accepted as the conventional method. However the method requires controlled heating for its success, and there are certain disadvantages, *e.g.* multistage staining, a cumbersome heating procedure and the discomfort caused by aerosols of phenol. There are several modified staining techniques for detection of AFB in sputum. Kinyoun is a well known method which requires high concentration of basic fuchsin and phenol or the addition of a detergent (tergitol no 7) there by avoiding the need for heat (Tanuphasiri and Kladphuang, 2002). In this study, an improved acid fast staining technique for staining of sputum, the MC staining method was described. This procedure used familiar Kinyoun solution, but the stages of staining were reduced and required no heating and combined counterstaining stages using Gabbet methylene blue solution- ultimately making the processing step faster, cheaper and safer. The method makes economic use of the laboratory and materials and these would be useful in large scale case finding programmes and laboratories with minimum facilities (Bhat and Bhat, 2000; Kocchar, 2002; and Tanuphasiri and Kladphuang, 2002). Another staining method

for detection of AFB is the FM that used AR or AP. The success of staining techniques depends on the ability of the dye to penetrate uniformly the cell wall of tubercle bacilli through their surface coating of waxy substance (Peterson *et al*., 1999). This study evaluates these four staining techniques used for preliminary diagnosis of TB.

In the present study, the total specimens studied were 1365 specimens from 500 clinically suspected PTB patients. During sample collection all the patients were advised to submit three sputum samples from each patient. But probably due to their personal reasons, 47 (9.4%) patients submitted only two samples and 44 (8.8%) patients submitted only single sample. 109 (21.8%) of the 500 patients were diagnosed as having PTB by the isolation of the organism from culture of sputum. The growth of organism in culture as *M. tuberculosis* was identified by cultural characteristics and acid fast staining. However positive culture confirmed by microscopy could not be tested with biochemical test and other nucleic acid probes for further identification.

In the present study, maximum number of TB cases was observed in the economically most productive age group of 15-24 years (26.1%), and more males were detected (24.2%), as compared to females (17.8%). Considering the gender in TB positive cases more males were found to be suffering from the disease than females (figure 1). This does not however reflect an increase in the occurrence of disease in males, since in the present study the attendance of females is lower than males (Table 1). TB was not diagnosed in the PTB suspects below 15 years. This data shows that the diagnosis of TB in childhood is surrounded by considerable uncertainty. The reason behind this may be that purulent sputum is not available from children. They usually shallow their sputum. but the gastric aspirates and laryngeal swabs are also not generally useful unless there is culture facility. So for the diagnosis of childhood TB, it should be done on clinical features based on constitutional symptoms and investigational reading. Tuberculin test if available may be useful in childhood diagnosis. This finding is in accordance to the previous report presented by Kabra *et al*. (2004).

In the study, the validity of four staining techniques was found by using culture as gold standard, for the diagnosis of TB. The sensitivity of ZN, Kinyoun, MC, and

Fluorochrome staining techniques were found to be 57.8%, 56%, 59.3% and 71.6% (Table 2, 3, 4). The sensitivity determination of ZN and MC stain was in accordance to those of Bhat and Bhat (2000); Kocchar (2002); and Tanuphasiri and Kladphuang (2002) who reported that no significant differences were found between the ZN and MC ($p>0.05$). In the study the results of the MC and ZN has the positive agreement of 98.6% which is in accordance with that of Tanuphasiri and Kladphuang (2002) who reported that the results of the MC and ZN has the close agreement of 97.2%. In the present study, Kinyoun method was reported to be slightly inferior to that of other three techniques and the finding was in agreement with the study of Somoskövi *et al*. (2001).

In the present study, the sensitivity of fluorescent staining technique (71.6%) was found significantly higher than that of other carbol fuchsin stained smear (Table 4). This finding contradict with the findings of Tanuphasiri and Kladphuang (2002) who reported that the Fluorochrome method was statistically less significant than that of ZN and MC ($p<0.05$). However this finding was strongly supported by several other reports of Somoskovi *et al*. (2001); Ulukanligil *et al*. (2000); Singh and Parija (1998); Jain *et al*. (2002); Pollock & Wieman (1977); and Githui *et al*. (1993), according to their studies the fluorescent staining techniques appeared to be more likely to detect TB smear which contains low density bacilli that are likely to be missed in carbol fuchsin stained smears.

The specificities of the ZN, Kinyoun, MC, and Fluorochrome staining techniques were found to be 98%, 98%, 98.2%, and 98%. The TN rate was very high in all four staining methods. These results are in agreement with the findings of Somoskovi *et al*. (2001); Ulukanligil *et al*. (2000); and Tanuphasiri and Kladphuang (2002). All of these studies reported that acid fast microscopy has high specificity. However, the specificity of smear examination methods should be interpreted with caution because it does not allow differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT).

Comparing to the culture results, the FP rate results obtained by four staining techniques were 2% in ZN, Kinyoun and Fluorochrome method; 1.8% in MC method (figure 2). These results are comparable with the several other findings of Somoskovi *et al*. (2001); Tanuphasiri and Kladphuang (2002); Ulukanligil *et al*. (2000); and Pollock & Wieman

(1977)**.** The FP results suggest that occasionally, a sputum specimen or a smear may contain particles that are acid fast: these particles may sometime resemble tubercle bacilli, *i.e.* MOTT or the precipitate of staining, which hampers reading. Because of FP results patients have to suffer from unnecessary therapy or prolonged hospital stay and further delays in the correct diagnosis and proper treatment of other diseases. In the study, FP rate was found similar in Fluorochrome method with other three staining techniques. But in several other studies claimed that higher FP rate might occur in Fluorochrome staining technique (Pollock & Wieman, 1977). Therefore, it is a good laboratory practice to confirm any smear-positive or doubtful result in newly diagnosed patients. Since, FP noted with the staining techniques was lower; a positive smear could be reliable as a good diagnostic indicator with these staining techniques.

In the study, it was also observed that very small percentage (1.2%) of smear positive specimen by all four staining techniques, were not able to be isolated on LJ medium, this may be due to the presence of non viable bacilli in sputum specimens received. Acid fast smear examination does not discriminate between viable and non viable bacilli, also tubercle bacilli and other mycobacteria (Gebre *et al*., 1995). This study is in accordance to the result obtained by Jain *et al*. (2002) who reported that 2-3% of AFB positive specimens, both by AR and ZN could not be confirmed by growth on LJ medium.

The FN rate by ZN, Kinyoun, MC stain and Fluorochrome stain in the study was found to be 42.2%, 44%, 40.4%, and 28.4% respectively (figure 3). Of the staining techniques, Kinyoun had highest FN rate while that of Fluorochrome result was lowest. The results are in accordance with the results of Somoskovi *et al*. (2001); Ulukanligil *et al*. (2000); and Tanuphasiri and Kladphuang (2002). FN results in the staining methods were commonly due to deficiencies in the preparation of the smear such as too little materials spread on the slide or too thin / thick smears. In the present study, concentrated sputum was divided into 5 aliquots, due to the fact, concentration of bacilli have may get varied. These data suggested that, a negative smear should be interpreted with caution because it does not rule out the active TB.

In the present study, higher percentages of patient were found to miss diagnosis when tested with sputum smear microscopy because of low sensitivity (figure 5). These data suggested that culture is the only definitive diagnosis of TB that depends on the isolation and identification of *M. tuberculosis*. Culture remained the gold standard diagnostic method for TB. Culture methods are highly sensitive and specific than microscopy for detection of bacilli, since approximately 10-100 mycobacteria per milliliter of sample is required for positive result while approximately 10^4 organism per ml of sputum is required to be seen by microscopic examination. But even when culture facilities are available, microbiological treatment is started on the basis of arbitrary clinical criteria and lack of response to other treatments. It has been shown that the proportion of positive sputum smear cases in the PTB-AIDS complex is even lower (Garay, 2000). So to provide the accurate diagnosis of PTB; a culture should always be requested concomitantly with AFB smear where the culture facilities are available. Culture requires at least a moderately well–equipped laboratory and necessarily lengthy time for its isolation and identification. So the cost and complexity associated with culture restricted its use only in major centers.

In the present study, the PPV and NPV were high for all four staining techniques (Table 2, 3 and 4). The PPV and NPV for ZN, Kinyoun, MC, Fluorochrome staining methods were 88.7%/ 89.3%, 88.4%/ 88.9%, 90.3%/ 89.7% and 90.7% and 92.5% respectively. These data suggested that all these four methods have sufficient validity to predict the presence or absence of the disease in TB prevalence population.

The accuracy of all four staining techniques results is expressed by ROC curve in figure 7. The ROC curve shows the relationship between sensitivity and specificity for respective staining techniques. Tests that perform less well have curves that fall closer to the diagonal running from lower left to upper right. ROC curves are particularly valuable way of comparing alternative tests for the same diagnosis. The overall accuracy of a test can be described as the area under the ROC curve; larger the area, better the test. In the present study the Fluorescent method has the largest area under the ROC curve of the four staining techniques and then the MC, ZN and Kinyoun respectively.

In the study, positive yield detected by fluorescent method (17.2%) was nearly close to that of culture (21.8%) than other staining methods (figure 6). From the results obtained in the present study, it can be said that FM was more sensitive and more reliable than the remaining other three techniques for demonstration of AFB. FM method has an added advantage of allowing a large number of sputum specimens to be examined in a given time as low power is used for examination. Moreover definite advantages of FM was that it enabled the detection of positive smears, which were over-looked with the carbol fuchsin stained smears containing low- density bacilli. So, the use of FM significantly increases the diagnostic value of the smear, particularly where there were low density bacilli (paucibacillary cases). Fluorochrome acid-fast microscopy is not only easy to perform and cost effective, but is currently the most rapid procedure for detecting AFB in clinical specimens and to screen for the most infectious cases of presumed tuberculosis. The fluorochrome-stained organisms can be seen at a lower magnification without the use of oil immersion. When lower magnifications are used, less microscopic viewing time is required, which creates the potential for decreasing the turnaround time needed to report microscopy results

FM method is quite economical in terms of both time and expenses in large laboratories handling large number of sputum specimens. Although the advantages of FM are the easiness in application, speed, and better contrast due to the dark background and higher sensitivity, it is not economical technique in rural areas of developing countries.

In the present study, among the carbol fuchsin stained smear, the MC stain has increased sensitivity (59.6%) than ZN (57.8%) and Kinyoun method (56.0%). The specificity of MC method (98.2%) was also higher than that of ZN (98%) and Kinyoun method (98%) and Fluorescent method (98%). The PPV and NPV were comparable with that of ZN, Kinyoun and Fluorochrome methods (Table 2, 3 and 4).

According to the present study and other previous studies of Bhat and Bhat (2000), Kocchar (2002) Tanuphasiri and Kladphuang (2002) and Somoskovi *et al*. (2001), it can be said that MC stain can be suitable and valuable alternative to that of ZN and Kinyoun because it has added advantage of ease, speed and is more economic than ZN and

Kinyoun and Fluorochrome methods. MC method can be very important diagnostic tool for the demonstration of AFB in developing countries with a large number of cases but have limited resources.

Of the staining techniques studied, Kinyoun method was inferior in term of sensitivity and specificity to those of other staining techniques, which were in agreement with the findings of Somosкövi *et al*. (2001).

From the present study, it can be found that diagnostic value of Fluorochrome method is more significant than the other three staining methods. It has higher sensitivity and specificity as well as positive and negative predictive value. So, the study concludes that Fluorochrome method is economical in terms of both time and expense and it can be recommended for laboratories handling large number of sputum specimens and the laboratories that can afford fluorescence microscope. Fluorochrome method is found to be more reliable among four techniques. But in the laboratories with minimum facilities and heavy work load, two steps simple MC method can be suitable as this method appeared to be more practical, rapid and effective. It can be concluded that MC method has been proved viable alternative to ZN and Kinyoun staining method for preliminary diagnosis of PTB.

To the best of our knowledge, this is the first study to evaluate four different staining techniques used in the primary diagnosis of PTB in Nepal. Today, attention has turned to NAA 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 PTB cases and for monitoring the progress of patients during treatment.

CHAPTER –VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

PTB is major public health agenda in developing countries. The highest priority for TB control is the identification and cure of infectious cases; i.e. patients with sputum smear positive PTB. Sputum smear examination is still the cornerstone of TB diagnosis in the developing world. The present study was conducted in Tuberculosis Research laboratory, BPKIHS, Dharan with the aim to evaluate four different staining techniques (Ziehl Neelsen, Kinyoun, Modified Cold and Fluorochrome stain) for the diagnosis of PTB and to determine the appropriate technique in the laboratory and public set up. In the study, culture was employed as a gold standard. The sample size in the study was 500 clinically suspected patients.

The major findings of the study are summarized as follows:

- Out of the 500 suspected patients, 59.6% was male and 40.4%was female, among whom 24.2% male and 17.8% female were suffered from TB. Considering the distribution of TB among genders, more males have found to be infected than females in all age groups.

- Maximum numbers of TB cases were observed in the economically most productive age group of 15-24 years (26.1%). Tuberculosis was not diagnosed in suspected cases below 15 years.

- One hundred nine patients (21.8%) were found positive by culture out of 500 suspected patients. The positive yields found by different staining techniques were 14.2% by ZN, 13.8% by Kinyoun, 14.4% by MC and 17.2% by Fluorochrome.

- 1.2% of PTB suspects were identified positive with all four staining methods but were not verified by culture.
- The sensitivity of ZN was 57.8%, Kinyoun was 56%, MC stain was 59.6% and Fluorochrome was 71.6%.
- The specificity was found to be 98% in ZN, Kinyoun and Fluorochrome and 98.2% in MC staining technique.
- The positive predictive value in different staining techniques was 88.7% in ZN, 88.4% in Kinyoun, 90.3% in MC and 90.7% in Fluorochrome.
- The negative predictive value in different staining techniques was 89.3% in ZN, 88.9% in Kinyoun, 89.7% in MC, and 92.5% in Fluorochrome.
- The false positive obtained in the study was 2% in ZN, Kinyoun, and Fluorochrome and 1.8% in MC staining techniques.
- The false negative obtained in the study was 42.2% in ZN, 44% Kinyoun, 40.4% MC, and 28.4% Fluorochrome staining techniques respectively.
- When the Kinyoun method was correlated with the ZN, the positive agreement was found to be 91.5%
- When the MC was correlated with the ZN, the positive agreement was found 98.6%.
- When the Fluorochrome method was correlated with the ZN, the positive agreement was found 98.6%.

7.2 RECOMMENDATIONS

i. In the laboratories handling large number of sputum specimens for TB diagnosis, Fluorochrome method is recommended.

ii. Use of MC staining technique is recommended over other techniques especially in rural areas and laboratories having limited facilities with heavy work load for TB diagnosis.

iii. Since all the staining techniques showed FN rate, culture of the sample is recommended. To provide the accurate diagnosis of pulmonary TB; a culture should always be requested concomitantly with AFB smear where the culture facilities are available.

iv. Proper diagnosis should be made on the combined basis of tuberculin test, AFB staining and culture.

v. In order to avoid FN rate and unnecessary burden to patients, molecular techniques like PCR technique should used for diagnosis.

CHAPTER – VIII

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APPENDIX - 1

MATERIALS AND CHEMICALS USED

1. REAGENTS:

Carbol fuchsin	S.D fine chem. Ltd, Biosar, India
Phenol	Merck Ltd, Mumbai, India Ltd
Ethanol	Merck Ltd, Mumbai, India
Absolute alcohol	Merck Ltd, Mumbai, India
Malachite green	Merck Ltd, Mumbai, India
Methylene blue	Merck Ltd, Mumbai, India
Auramine	Hi Media laboratories, Pvt Ltd, India
Sodium hydroxide pellets	Merck Ltd, Mumbai, India
Dehydrated alcohol	Bengal chemicals & pharmaceutical Ltd, Calcutta,
Potassium permanganate	
Hydrochloric acid	LOBA CHEMIE Pvt, Ltd, India
Sulphuric acid	Merck Ltd, Mumbai, India

2. MEDIA :

Magnesium citrate	Merck Ltd, Mumbai, India
Magnesium sulphate, pure	Merck Ltd, Mumbai, India
Potassium dihydrogen	
Phosphate	Merck Ltd, Mumbai, India
L - Asparagine monohydrate	Lancaster, Eastgate, England
Glycerol (98% purified)	Merck Ltd, Mumbai, India

3. MATERIALS:

Lysol	Overseas chemical industries, Calcutta, India
Xylene	Merck Ltd, Mumbai, India
Methylated spirit	Merck Ltd, Mumbai, India

Immersion oil	LOBA CHEMIE Pvt, Ltd, India
Centrifuge tubes(15 ml)	Tarson, Graduated, Radiation sterilized
Blue star, microslides 25cm x 75cm,	Polar industrial corporation
Gloves	Mexpo International, Malaysia
Slide box	
Bunsen burner	
Diamond pen	
Filter paper	

4. EQUIPMENTS:

Oven	Gallenkamp, England
Binocular microscope	Olympus
Incubator	York scientific industries Pvt Ltd.
Bio-safety cabinet	Deepak MediTech Pvt Ltd, dehli
Research centrifuge	REMI Instruments Ltd, Mumbai India.
Inspissator	Chemical & Instrumentals Corporation, India
Fluorescence microscope	
Refrigerator	
Distilled water plant	

APPENDIX 2

PROFORMA

Lab No: **Date:**
S. No:

A) Identification of patient:

Name: **Age:** **Sex: M/F**
Education: primary /secondary/intermediate/bachelor/master
Occupation
Socio-economic status: **Religion:**
H/M/B/C/Others

B) Address:

Zone: **District:**
VDC/Municipality: **Ward no:**

C) History:

Symptoms	y/n	Duration of symptoms (days)	remarks
Cough	y/n		
Expectoration	y/n		
Hemoptysis	y/n		
Shortness of breath	y/n		
Anorexia	y/n		
Weight loss	y/n		
Fever	y/n		
Chest pain	y/n		

APPENDIX-3

STAINING REAGENT AND MEDIA PREPARATION:

A. Staining reagents

1. Ziehl-Neelsen stain

i) Ziehl-Neelsen carbol fuchsin

Alcoholic basic fuchsin (saturated solution)

Ingredients	composition
Basic fuchsin	3g
Ethyl alcohol, 95%	100ml

Phenol, 5%

Ingredients	composition
Phenol	5g
Distilled water	100ml

ii) Decolorizing agents

Acid-alcohol

Ingredients	composition
Concentrated Hcl	3ml
95% ethyl alcohol	97ml

iii) Counter stain

Ingredients	composition
Malachite green	0.3g
Distilled water	100ml

2. Fluorochrome stain

i) Auramine O Fluorochrome stain

Alcoholic Auramine solution

Ingredients	composition
Auramine O	0.1g

Ethyl alcohol, 95%	10ml

Phenol

Ingredients	**composition**
Phenol crystals	3g
Distilled water	87ml

ii) Decolorizing agent

1% acid-alcohol

Ingredients	**composition**
Concentrated Hcl	20ml
Spirit, methylated industrial	1980ml

iii) Counter stain

0.1% potassium permanganate

Ingredients	**composition**
Potassium permanganate	2g
Distilled water	2 litres

3. Kinyoun stain

i) Kinyoun carbol fuchsin

Ingredients	**composition**
Basic fuchsin	4g
Ethyl alcohol	20ml
Phenol crystals	8g
Distilled water	100ml

ii) Decolorizing agent and counter stain same as Ziehl-Neelsen stain.

4. Modified cold stain

i. Primary stain is Kinyoun carbol fuchsin

ii. Gabbet methylene blue stain

Ingredients	**composition**
Methylene blue	1g
Absolute alcohol	30ml

Concentrated sulfuric acid	20ml
Distilled water	50ml

B. 4% Sodium hydroxide (NaOH) solution

Ingredients	composition
Sodium hydroxide pellets (analytical grade)	4g
Distilled water	100 ml

Sodium hydroxide was dissolved in distilled water, then distributed in conical flasks and sterilized by autoclaving at 121^0C for 20 minutes.

Sterile distilled water

Five hundred milliliter distilled water was sterile by autoclaving in flasks at 121^0C for 20 minutes.

C. CULTURE MEDIA (Lowenstein Jensen media)

Preparation of the International Union Against Tuberculosis Lowenstein-Jensen medium (IUTM). (IUATLD-1998)

a) IUTM base composition

Ingredients	composition
L-Asparagine	2.25g
Potassium phosphate,dibasic, anhydrous	1.50g
Magnesium sulphate $7H_2O$	0.4g
Magnesium citrate	0.6g
Malachite green	0.25g

b) Preparation of Lowenstein-Jensen medium

Ingredients	composition
IUTM base	4.5g
Glycerol	7.5 ml
Distilled water	360 ml

Procedure:

- About 360 ml mineral salt solution with malachite green was prepared, and then it was autoclaved at 121^0C for 15 minutes for sterilization.
- Fresh hen's eggs were soaked in soap water for 30 minute, and then it was scrubbed with 70 % ethanol to clean them.
- Eggs were broken, white and yolk were homogenized in sterile blender, and then drained through sterile gauge.
- To the autoclaved salt solution about 625 ml of homogenized whole eggs were added, and then after mixing thoroughly it was dispensed aseptically.
- The complete medium was dispensed in 6-8 ml volume in sterile Mackintosh bottle and then it was inspissated at 85^0C for 50 minute in a slanted position.

APPENDIX-4

Statistical analysis

Gabbet (+=1, -=2) * culture Crosstabulation

			culture		Total
			1	2	
Gabbet (+=1, -=2)	1	Count	65	7	72
		% within Gabbet (+=1, -=2)	90.3%	9.7%	100.0%
		% within culture	59.6%	1.8%	14.4%
	2	Count	44	384	428
		% within Gabbet (+=1, -=2)	10.3%	89.7%	100.0%
		% within culture	40.4%	98.2%	85.6%
Total		Count	109	391	500
		% within Gabbet (+=1, -=2)	21.8%	78.2%	100.0%
		% within culture	100.0%	100.0%	100.0%

Ziehl-Neelson * culture Crosstabulation

			culture		Total
			1	2	
Ziehl-Neelson	1	Count	63	8	71
		% within Ziehl-Neelso	88.7%	11.3%	100.0%
		% within culture	57.8%	2.0%	14.2%
	2	Count	46	383	429
		% within Ziehl-Neelso	10.7%	89.3%	100.0%
		% within culture	42.2%	98.0%	85.8%
Total		Count	109	391	500
		% within Ziehl-Neelso	21.8%	78.2%	100.0%
		% within culture	100.0%	100.0%	100.0%

Flourchrome * culture Crosstabulation

			culture		Total
			1	2	
Flourchrome	1	Count	78	8	86
		% within Flourchrome	90.7%	9.3%	100.0%
		% within culture	71.6%	2.0%	17.2%
	2	Count	31	383	414
		% within Flourchrome	7.5%	92.5%	100.0%
		% within culture	28.4%	98.0%	82.8%
Total		Count	109	391	500
		% within Flourchrome	21.8%	78.2%	100.0%
		% within culture	100.0%	100.0%	100.0%

kinyoun * culture Crosstabulation

			culture		Total
			1	2	
kinyoun	1	Count	61	8	69
		% within kinyoun	88.4%	11.6%	100.0%
		% within culture	56.0%	2.0%	13.8%
	2	Count	48	383	431
		% within kinyoun	11.1%	88.9%	100.0%
		% within culture	44.0%	98.0%	86.2%
Total		Count	109	391	500
		% within kinyoun	21.8%	78.2%	100.0%
		% within culture	100.0%	100.0%	100.0%

Gabbet (+=1, -=2) * Ziehl-Neelson Crosstabulation

			Ziehl-Neelson		Total
			1	2	
Gabbet (+=1, -=2)	1	Count	70	2	72
		% within Gabbet (+=1, -=2)	97.2%	2.8%	100.0%
		% within Ziehl-Neelson	98.6%	.5%	14.4%
	2	Count	1	427	428
		% within Gabbet (+=1, -=2)	.2%	99.8%	100.0%
		% within Ziehl-Neelson	1.4%	99.5%	85.6%
Total		Count	71	429	500
		% within Gabbet (+=1, -=2)	14.2%	85.8%	100.0%
		% within Ziehl-Neelson	100.0%	100.0%	100.0%

Flourchrome * Ziehl-Neelson Crosstabulation

			Ziehl-Neelson		Total
			1	2	
Flourchrome	1	Count	70	16	86
		% within Flourchrome	81.4%	18.6%	100.0%
		% within Ziehl-Neelson	98.6%	3.7%	17.2%
	2	Count	1	413	414
		% within Flourchrome	.2%	99.8%	100.0%
		% within Ziehl-Neelson	1.4%	96.3%	82.8%
Total		Count	71	429	500
		% within Flourchrome	14.2%	85.8%	100.0%
		% within Ziehl-Neelson	100.0%	100.0%	100.0%

KINYOUN * Ziehl-Neelson Crosstabulation

			Ziehl-Neelson		Total
			1	2	
KINYOUN	1	Count	65	4	69
		% within KINYOUN	94.2%	5.8%	100.0%
		% within Ziehl-Neelson	91.5%	.9%	13.8%
	2	Count	6	425	431
		% within KINYOUN	1.4%	98.6%	100.0%
		% within Ziehl-Neelson	8.5%	99.1%	86.2%
Total		Count	71	429	500
		% within KINYOUN	14.2%	85.8%	100.0%
		% within Ziehl-Neelson	100.0%	100.0%	100.0%

APPENDIX – 6

DEFINATIONS OF TERMINOLOGY USED IN THE STUDY

I Site of TB disease

- PTB refers to involving the lung parenchyma. Therefore, tuberculous intrathoracic lymphadenopathy (mediastinal and/or hilar) or tuberculous pleural effusion, without radiographic abnormalities in the lungs, constitue a case of extra pulmonary TB (EPTB).
- A patient with both pulmonary and extra pulmonary TB constitutes a case of PTB.
- A case definition of extra pulmonary case with several sites affected depends on the site representing the most severe form of disease.

II New case

- A patient who has never had treatment for TB or who has taken ATT drugs for less than four weeks.

III Smear Positive PTB: Presence of AFBs on sputum microscopy

IV Smear Negative PTB: Absence of AFBs on sputum microscopy

V Positive case: A patient with at least one sputum specimen positive for AFB by microscopy which is culture positive for *M. tuberculosis*

VI Sensitivity: It refers to the proportion of people who have positive test results

Sensitivity = true positive/ (true positive + false negative)

VII Specificity: It refers to the proportion of people who do not have disease when test is negative

Specificity= true negative/ (true negative + false positive)

VIII Positive predictive value: Probability that the patient has the disease when restricted to those patients who test positive

Positive predictive value = true positive/ (true positive + false positive)

IX Negative predictive value: Probability that the patient does not have disease when restricted to those patients who test negative

Negative predictive value = true negative/ (true negative + false negative)