AN EVALUATION OF 5% NAOCl MICROSCOPY METHOD FOR THE LABORATORY DIAGNOSIS OF PULMONARY TUBERCULOSIS

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(Medical)

By
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RECOMMENDATION

This is to certify that **Mr. Suman Lama** has worked under our supervision and guidance on the thesis entitled **"An evaluation of 5% NaOCl microscopy method for the laboratory diagnosis of pulmonary tuberculosis".** To the best of our knowledge this is an original research work of his and has not been submitted for any other degrees.

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ABSTRACT

Direct sputum smear microscopy forms the mainstay for pulmonary tuberculosis case-finding in the resource-poor countries like Nepal. However, this method is hampered by the lack of sensitivity. Bleaching of sputum with sodium hypochlorite and concentration of mycobacteria prior to Ziehl-Neelsen staining method is a possible means of improving the sensitivity of direct microscopy. Therefore, this study was performed in National Tuberculosis Centre, Thimi, Bhakatapur, Nepal with an objective to evaluate 5% NaOCl microscopy method for the primary diagnosis of pulmonary tuberculosis.

A total 475 sputum were collected from 159 suspected pulmonary tuberculosis patients. Direct smears were prepared from all sputum. 'On-spot' sputum was divided equally into three tubes (A, B and C). Tube A and B were treated by NaOCl-centrifugation method and NaOCl-sedimentation method respectively, and ZN smears were prepared. Tube C was treated by Modified Petroff's method for culture. NaOCl-centrifuged and NaOCl-sedimented 'on-spot' sputum showing positive direct smears were also used for culture to ascertain the sterilizing activity of NaOCl. Culture method was employed as a gold standard for pulmonary tuberculosis diagnosis.

Direct 'on-spot' sputum Ziehl-Neelsen smears, NaOCl-centrifuged Ziehl-Neelsen smears and NaOCl-sedimented Ziehl-Neelsen smears had 17.7%, 16.3% and 10.1% positivity respectively compared to 14.5% of the standard three smears strategy. There was a statistically significant increase in the number of acid-fast bacilli positive sputum samples by NaOCl methods ($P<0.05$). In addition, there was no statistical significant between two NaOCl methods ($P>0.05$). With reference to culture, sensitivity of direct 'on-spot' sputum Ziehl-Neelsen smears, NaOCl-centrifuged Ziehl-Neelsen smears and NaOCl-sedimented Ziehl-Neelsen smears were found to be 35.1%, 67.5% and 62.1% compared to 54% of standard three smears strategy. The specificity was found to be same i.e. 97.4% for all methods. The positive and negative predictive values were found to be 81.2%/82.3% for direct 'on-spot' sputum Ziehl-Neelsen smears, 89.2%/90.4% for NaOCl-centrifuged Ziehl-Neelsen smears, 88.4%/89% for NaOCl-sedimented Ziehl-Neelsen smears and 87%/87% for standard three smears strategy. No growth of *Mycobacteria tuberculosis* was seen in culture media (Lowenstein-Jensen) inoculated with NaOCl-centrifuged and NaOCl-sedimented 'on-spot' sputum showing the biocidal activity of NaOCl.

This study indicates that 5% NaOCl microscopy method i.e. NaOCl-centrifuged Ziehl-Neelsen smears and NaOCl-sedimented Ziehl-Neelsen smears could be alternative to direct microscopy and the application of these methods would make a positive impact on the effectiveness of TB control programs.

Keywords: Tuberculosis, *M. tuberculosis*, Sputum smear microscopy, Bleach digestion, Ziehl-Neelsen.

LIST OF CONTENTS

ABBREVIATION

AFB	Acid Fast Bacilli
AIDS	Acquired Immuno Deficiency Syndrome
ALA	American Lung Association
AMTD	Amplified Mycobacterium Tuberculosis Direct Test
CD4+	Cluster of Differentiation 4 Glycoprotein
CD8+	Cluster of Differentiation 8 Glycoprotein
CD95	Cluster of Differentiation 95 Glycoprotein
CDC	Centers for Disease Control and Prevention
CR	Complement Receptor
DOTS	Direct Observed Treatment Strategy
ELISPOT	Enzyme-Linked Immunospot
EPTB	Extra Pulmonary Tuberculosis
FN	False Negative
FP	False Positive
GLC	Gas Liquid Chromatography
HCW	Healthcare Worker
HIV	Human Immuno Deficiency Virus
HPLC	High Performance Liquid Chromatography
IFN-γ	Interferon-Gamma
IUATLD	International Union Association of Tuberculosis and Lung Disease
LAI	Laboratory-Acquired Infection
LAM	Lipoarabinomannan
LJ	Lowenstein-Jensen
LTBI	Latent Tuberculosis Infection

MGIT	Mycobacteria Growth Indicator Tube
MHC	Major Histocompability Complex
MODS	Microscopic Observation Broth-Drug Susceptibility Assay
MOTT	Mycobacterium Other Than Tuberculosis
NAA	Nucleic Acid Amplification
NPV	Negative Predictive Value
NTC	National Tuberculosis Centre
NTP	National Tuberculosis Program
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Luekocytes
PPV	Positive Predictive Value
PRA	PCR Restriction Enzyme Analysis
PTB	Pulmonary Tuberculosis
SAARC	South Asian Association for Regional Cooperation
SEAR	South-East Asia Region
SSM	Sputum Smear microscopy
SS-	Sputum Smear Negative
SS+	Sputum Smear Positive
STC	SAARC Tuberculosis Centre
TB	Tuberculosis
TLC	Thin Layer Chromatography
TH	T Helper Cell
WHO	World Health Organization
ZN	Ziehl Neelsen

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF APPENDIX

CHAPTER- I

1. INTRODUCTION

Tuberculosis (TB) continues to be a devastating disease worldwide and is believed to be present in about one third of the world's population. It is estimated that about 8 million new cases of TB and about 2 million deaths from this disease occur annually around the world (Pal and O'Brien, 2006). Approximately 95% of cases and 98% of deaths occur in the developing world with the greatest burden in Sub-Saharan Africa and South-East Asia (Montoro and Rodriguezn, 2007). Globally, TB causes more adult deaths than any other single infectious disease (Lawson *et al.,* 2006). Despite the widespread implementation of World Health Organization's (WHO) strategy, the case detection rate has remained low. This inadequate case detection has become the primary impediment to disease control and the global target for case detection has been not met yet.

DOTS (Direct Observed Treatment Strategy), WHO global strategy for TB control, relies upon sputum smear microscopy (SSM) for detecting the infectious cases of pulmonary TB (PTB). This usually involves suspected patients submitting three sputum over the course of two days. It is estimated that 60-70% of all TB cases are diagnosed by means of SSM (Githui *et al.,* 1993). Detection of smear positive case is the highest priority in a TB control program because these cases are highly infectious and contribute substantially to the transmission of PTB (Harries *et al.,* 2005). The finding of acid-fast bacilli (AFB) in sputum smear establishes a presumptive diagnosis of PTB and is crucial to limit person to person spread of disease, to assess the degree of infectivity of disease and to guide treatment (Behr *et al.,* 1999). Thus, SSM is the most efficient way of detecting the sources of transmission and represents one of the five pillars of DOTS (Van Duen *et al.,* 2002; WHO, 2003). WHO recommended definition for smear positive cases of PTB requires at least two positive smears for AFB or where culture facilities are available, one positive smear for AFB with one culture positive for *Mycobacterium tuberculosis* (Lawson *et al.,* 2006).

Since the Koch's discovery of tubercle bacilli in 1882, microscopic detection of bacilli in clinical specimens has remained cornerstone of TB diagnosis and is equivalent of confirmed diagnosis in low-income and middle-income countries (Hopewell *et al.,* 2006). SSM has the advantage of being simple, inexpensive and relatively rapid to perform. The specificity of

SSM is excellent for AFB but the sensitivity is not optimal when used in a control program. The higher concentration of bacilli, typically over 10,000 bacilli per ml of sputum, must be present to detect in SSM. The sensitivity of SSM is also influenced by numerous factors, such as prevalence and severity of the disease, type of specimen, quality of specimen collection, method of processing (direct or concentrated), g factor for centrifugation, staining artifacts, staining technique and quality of examination (Saceanu *et al.,* 1993). The sensitivity of SSM is further undermined in areas with high HIV prevalence since HIV co-infection, probably through modifying the TB disease process, increases the proportion of patients with culture positive and smear negative TB (Bruchfeld *et al.,* 2000; Perkins and Cunningham, 2007).

Generally, preparation of direct smear from sputum is considered hazardous for laboratory technician working in low-income and middle-income countries with limited facilities. Good laboratory practices have to be followed while making smears to avoid laboratory-acquired TB infection (Selvakumar *et al.,* 2006). If the performance and sensitivity of SSM could be improved, it has the potential to become even more valuable tool for National Tuberculosis Control Programs (NTP) around the world. Several methods have been used to improve the performance and sensitivity of SSM. A variety of chemical and physical methods have been used to concentrate tubercle bacilli by means of gravitational force or filtration, or to improve their dispersion in sputum (Perkins and Cunningham, 2007). However, methods aiming to concentrate tubercle bacilli by digestion of sputum with bleach (NaOCl) and subsequently concentration by centrifugation or sedimentation have received much more attention than others (Lawson *et al.,* 2006).

Several studies reported that the sensitivity of SSM can be significantly augmented after liquefaction of sputum with NaOCl followed by concentration. Moreover, NaOCl is a potent disinfectant which kills mycobacteria and eliminates the risk of laboratory-acquired TB infection, a risk that cannot be neglected, especially in the laboratories with inadequate safety standards (Angeby *et al.,* 2000). The additional cost of NaOCl digestion to current cost of SSM is likely to be negligible (Yassin *et al.,* 2003).

The definitive diagnosis of TB can only be performed by culturing *M. tuberculosis*. The inoculation of concentrated bacilli from processed clinical specimens on solid media (like Lowenstein-Jensen media) is a standard approach for confirmation of TB (Kar *et al.,* 2003). Culture method is more sensitive than SSM as it can detect 10-100 mycobacteria per ml of

sputum. Therefore, culture is deemed to be the gold standard for diagnosis of TB (Glassroth, 1993). 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 and middle-income countries (Parekh and Kar, 2003).

In developing countries like Nepal, SSM for detection of AFB in patients suggestive of PTB forms the cornerstone of NTP. In SSM, Ziehl-Neelsen (ZN) staining is the most commonly used technique because of its simplicity and low cost. Culture and drug-sensitivity testing are available only at the central level. Although many advances in techniques for diagnosis of PTB have been made, the use of SSM still remains the most available and commonly used method in Nepal. Over-burden SSM services in Nepal urgently need methodologies that are safe, simple and give rapid results. Therefore, this study is set out to compare NaOCl-digested smears with direct ZN smears and culture in patients presenting with symptoms suggestive of PTB to Nation Tuberculosis Centre (NTC). If a single NaOCl-digested sputum ZN smear could replace the examination of the standard three smears and provide a one-step diagnosis, it could make a significant contribution to improve case detection rate.

CHAPTER - II

2. OBJECTIVES

2.1 General objective

- To find the potential of NaOCl microscopy method for the laboratory diagnosis of pulmonary tuberculosis.

2.2 Specific objectives

- To compare the smear positivity achieved by direct 'on-spot' Ziehl-Neelsen smears, NaOCl-centrifugation method, NaOCl-sedimentation method and standard three smears strategy.
- To compare the smear positivity achieved by NaOCl-centrifugation method and NaOCl-sedimentation method with that of WHO case definition.
- To evaluate the safety of NaOCl.
- To compare sensitivity and specificity of direct 'on-spot' Ziehl-Neelsen smears, NaOCl-centrifugation method, NaOCl-sedimentation method and standard three smears strategy.
- To compare positive and negative predictive value of direct 'on-spot' Ziehl-Neelsen smears, NaOCl-centrifugation method, NaOCl-sedimentation method and standard three smears strategy.

CHAPTER – III

3. LITERATURE REVIEW

TB is a common and deadly infectious disease caused by mycobacteria, mainly *M. tuberculosis*. TB most commonly affects the lungs in more than 80% of cases (these are called PTB). TB spreads from the primary lung lesions to other parts of the body (like meninges, kidneys, genital tract, bone and joints) *via* the blood stream, lymphatic system and bronchial system or by direct extension. In this way, it may affect any organ causing extra-pulmonary TB (EPTB). Other mycobacteria such as *M. bovis*, *M. africanum*, *M. canetti* and *M. microti* can also cause TB but these species do not usually infect healthy adults (Raviglione and O'Brien, 2004).

3.1 History

The word 'tuberculosis' is a derivative of Latin word 'tubercula' which means a small lump. Several names have been used to refer to TB such as phthisis, lung sickness, consumption, lung vulgaris, mesenteric disease, Pott 's disease, scrofula, king's evil, white plague and white swelling, in a year gone by (Leao and Portaels, 2007).

TB has a long history. It is presumed that the genus Mycobacterium originated more than 150 million years ago. An early progenitor of *M. tuberculosis* was probably contemporaneous and co-evolved with early hominids in East Africa, 3 million years ago. The modern members of *M. tuberculosis* complex seem to have originated from a common progenitor about 15,000-35,000 years ago. TB has been present in human since antiquity. TB was documented in Egypt, India and China as early as 5,000, 3,300 and 2,300 years ago respectively. Typical skeletal abnormalities, including pott's deformities, were found in Egyptian and Andean mummies, and were also depicted in the early Egyptian and pre-Colombian art (Leao and Portaels, 2007). The earliest unambiguous detection of *M. tuberculosis* is in the remains of long-horned Pleistocene bison dated 17,870 +/- 230 years ago (Rothschild *et al.,* 2001). However, whether TB originated in cattle and then transferred to human or diverged from common ancestor is currently unclear. Skeletal remains show prehistoric human had TB and

tubercular decay has been found in the spines of mummies from 3000-2400 BC (Zink *et al.*, 2003).

Phthisis (meaning consumption, to waste away) is a Greek term for TB. Around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times, most commonly occurred between 18 and 35 years of age, involving coughing up blood and fever, which was almost always fatal. Clarissimus Galen defined phthisis as an ulceration of the lungs, chest or throat accompanied by cough, low fever and wasting away of the body because of pus. He described it as a disease of malnutrition (Pease, 1940).

In the 18th century, TB was sometimes regarded as vampirism. Precise pathological and anatomical descriptions of the disease began to appear in the 17th century. Franciscus Sylvius de la Boe was the first person to identify the presence of actual tubercles as a consistent and characteristic change in the lungs and in the other areas of consumptive patients. The English physician Richard Morton confirmed that tubercles were always present in TB of the lungs. Gaspard Laurent Bayle definitely proved that tubercles were not products or results but the very cause of the illness. The name 'tuberculosis' appeared in the medical language at 1774-1818 AD in connection with Bayle's theory. More precisely, the name 'tuberculosis' was first coined by Johann Lukas Schonlein, German Professor of Medicine, to describe diseases with tubercles in 1839 AD. The English physician Benjamin Marten was the first to conjecture that TB could be caused by 'minute living creatures' which once gained entry to the body could generate the lesions and symptoms of phthsis. He also stated that consumption may be caught by a sound person by lying in the same bed, eating and drinking or by talking together so close to each other as to draw in part of the breath a consumptive patient emits from the lungs. The French doctor Jean-Antoine Villemin demonstrated that consumption could be passed from human to cattle and from cattle to rabbits, and postulated that a specific microorganism caused the disease. William Budd also concluded that TB was spread through society by specific germs. The etiological agent of TB, *M. tuberculosis* was identified and described by Robert Koch on March 24, 1882 AD (Leao and Portaels, 2007).

3.2 Epidemiology

3.2.1 TB worldwide

TB is a major threat to the global health, infecting one third of the global population (2 billion people). It is estimated that 8.8 million new cases of TB occurred in 2005 (136/100,000 population) and about 3.9 million cases (60/100,000 population) were sputum smear positive (SS+). There were 14.05 million prevalent cases (217/100,000), of which 6.1 million were SS+ (95/100,000 population). It is estimated that 1.6 million (24/100,000) death results from TB in 2005, including those co-infected with HIV (WHO, 2007).

Worldwide, 1 person out of 3 is infected with *M. tuberculosis* (Montoro and Rodriguezn, 2007). TB accounts for 2.5% of the global burden of disease and is the commonest cause of death in young women, killing more women than all causes of maternal mortality combined (STC, 2005). Effective drugs to treat and cure the disease have been available for more than 50 years, yet every 15 seconds, someone in the world dies from TB. Even more alarming, a person is newly infected with *M. tuberculosis* every second of every day. Left untreated, a person with active TB will infect an average of 10 to 15 other people every year (Dye, 2006; WHO, 2006).

3.2.2 TB in Asia

Asia has the highest burden of TB in the world, every 30 seconds a person dies of TB (WHO, 2006). In 2005, Asia had the highest TB case rate (25.5/100,000 population) (ALA, 2006). The majority of patients with TB live in the most populous countries of Asia: Bangladesh, China, India, Indonesia and Pakistan, together account for half (48 %) of the new cases that arise every year (Montoro and Rodriguezn, 2007).

3.2.3 TB in South-East Asia Region (SEAR)

WHO estimated that the largest number of new TB cases in 2005 occurred in the SEAR which accounted for 34% of the incident cases globally. In 2005, of the estimated 5 million prevalent cases of TB, almost 3 million were new cases, reflecting the incidence rate of 182 per 100,000 population. Over half a million people continue to die of the disease every year in this region, one person every minute (WHO, 2006).

3.2.4 TB in South Asian Association for Regional Cooperation (SAARC)

SAARC region bears 28.31% of global TB burden with approximately 2.5 million new TB cases and 0.6 million deaths per year. More than 1.12 million new persons develop infectious SS+ PTB each year in this region (STC, 2005). India, Bangladesh, Pakistan and Afghanistan have the largest number of TB patients, occupying the 1st, 6th, 7th and 17th position in the list of 22 high-burden TB countries (Montoro and Rodriguezn, 2007).

3.2.5 TB in Nepal

Nepal is among the intermediate TB burden countries in SEAR and ranked 27 globally by estimated number of cases (WHO, 2006). In Nepal, about 45% of the total population is infected with TB, of which 60% were adults. Every year about 40,000 people develop TB. Nearly half of them i.e. 20,000 have infectious SS+ PTB. It is estimated that about 5,000-7,000 people die of TB each year (NTC, 2007). In a study conducted by Maharjan *et al.,* it was estimated that the annual rate of TB infection is about 3% in terai, 1.5% in hilly areas, 4% in urban areas and less than 1% in mountain areas (Maharajan *et al.,* 2003).

3.2.6 Epidemiology of laboratory-acquired TB

Staff working in microbiological diagnostic and research laboratories is likely to be exposed to infection risk with pathogens. Yearly, the incidence of TB among laboratory staff is about 0.3 per 1000 people. More recently, it was confirmed that TB remained in the so-called 'Top-10' laboratory-acquired infection (LAI) (Herman *et al.,* 2006). LAI of TB mainly occurred *via* aerosols or less frequently by accidental inoculation. More than 30 years ago, it was clearly documented that the incidence of TB in laboratory workers was nine times higher than in the community (Rattan *et al,* 1994). In 1957, Merger reported that the incidence was twenty eight times higher in laboratory workers than in the general population (Merger, 1957). Reid also reported that those laboratory workers who handled fresh materials were two to five times more likely to become infected compared to matched controls. The incidence of TB among personnel manipulating samples potentially contaminated with *M. tuberculosis* is three to nine times higher than that observed in people not working with such tubercle bacilli samples (Shinnick and Good, 1995).

A study showed that behavioral factors are important in the contribution of LAI of TB. It was observed that 80% of all accidents were due to human errors and 20% were due to equipment

problems. Now a day, even if equipment troubles were partially solved by the adoption of appropriate safety equipment in many diagnostic and research laboratories, behavioral factors may be a source of concern (Herman *et al.,* 2006).

3.3 Transmission of TB

TB transmission occurs by airborne spread of infectious droplet nuclei, 1-5μm in diameter that contains 1-3 tubercle bacilli each, generated by coughing, sneezing, singing and other forced respiratory maneuvers. In most instances, only one such droplet nucleus is believed to be responsible for establishing infection in the host. Tubercle bacilli that lodged on fomites do not constitute a significant source of infection since they die quickly through the action of drying, heat or sunlight. The source of infection is person with PTB. Patients who are SS+ infect many of their close contacts whereas those who are SS- and culture positive infect far fewer contacts than SS+ patients (Long and Jessamine, 2000). The relative transmission rate of SS- patients compared with SS+ patients was calculated as 0.22 and accounted for 17% of all transmissions (Kanaya *et al.,* 2001; Long and Jessamine, 2000).

The probability of transmission of TB from one person to another person depends upon the concentration of infectious droplets expelled, duration of exposure (the closer the proximity and the longer the duration of exposure, the higher the risk is for being infected), effectiveness of ventilation, the inadequate cleaning and disinfection of medical equipments, and improper procedures for handling specimens (CDC, 2007; Jensen *et al.,* 2005).

3.4 Risk factor for TB

3.4.1 Persons at high-risk for exposure to and infection with *M. tuberculosis*

Characteristics of persons exposed to *M. tuberculosis* that might affect the risk for infection are not well defined. According to Center for Disease Control and Prevention (CDC), Advisory Council for the Elimination of TB, the following persons are at higher risk for exposure to and infection with *M. tuberculosis* (Jensen *et al.,* 2005).

1. Persons who are foreign-born, including children, recently arrived (within 5 years) from geographic areas with a high incidence or prevalence of TB disease.

2. Residents and employees of congregate settings (e.g. correctional facilities, long-term care facilities and homeless shelters).
3. Healthcare workers (HCW) who serve high-risk patients.
4. HCW with unprotected exposure to patient with TB disease before the identification and correct airborne precautions of the patient.
5. Populations who are defined locally as having an increased incidence of TB disease.
6. Infants, children and adolescents exposed to adults in high-risk categories.

3.4.2 Persons at high-risk for progression from latent TB infection (LTBI) to TB disease

Following primary TB infection, the lifetime cumulative risk for the development of active TB is generally estimated to be 10%. Half of these cases will occur in the first 2 to 3 years after infection. Certain factors increase the risk of TB reactivation because of diminished local or systemic immunity (Jensen *et al.,* 2005; Menzies and Pourier, 2000).

1. Persons infected with HIV.
2. Persons infected with *M. tuberculosis* within the previous 2 years.
3. Persons with the clinical or immunocompromising conditions like chronic renal failure, diabetes mellitus, silicosis, leukemias, lymphomas etc.
4. Persons with a history of untreated or inadequately treated TB disease including persons with chest radiograph findings consistent with previous TB disease.
5. Persons who inject illict drugs or other locally identified high-risk substance users.

3.5 Virulence factors and virulence mechanisms

M. tuberculosis does not possess the classic bacterial virulence factors like toxins, capsules and fimbriae. However, numbers of structural and physiological properties of the bacterium are beginning to be recognized for their contribution to bacterial virulence and the pathology of TB. Cord factor and sulphatides are the recognized virulent factors of *M. tuberculosis* (Jolik *et al.*, 1992).

M. tuberculosis has the special mechanisms for cell entry. The bacilli can bind directly to mannose receptors on macrophages *via* the cell wall associated mannosylated glycolipid, LAM or indirectly *via* certain complement receptors (like CR 1, CR 2, CR 3 and CR 4) or Fc

receptors or surfactant proteins (Pando *et al.*, 2007). It can grow intracellularly and evades the immune system. Once it is phagocytosed, it can inhibit phagosome-lysosome fusion (Goldsby *et al.*, 2003). It also interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by down regulating the oxidative cytotoxic mechanism or bypassing the activation of a respiratory burst (Raja, 2004). Slow generation time of *M. tuberculosis* also cause immune system not to readily recognize the bacilli or not be triggered sufficiently to eliminate them.

Antigen 85 complex is a group of proteins secreted by *M. tuberculosis* that are known to bind fibronectin which may aid in walling off the bacteria from the immune system and may facilitate tubercle formation. The cord factor is known to be an inhibitor of PMN migration. High lipid concentration in cell wall accounts for resistance to osmotic lysis *via* complement deposition and attack by lysozyme.

3.6 Pathogenesis

TB infection begins when the droplet nuclei containing tubercle bacilli lodge in the terminal alveoli of lungs where they invade and replicate within alveolar macrophage. The additional inflammatory cells are attracted to that areas, which lead to formation of a small inflammatory lesions i.e. tubercle (Cheesbrough, 2002). In the early weeks of infection, some infected macrophages are borne to regional lymph nodes (e.g. hilar, mediastinal). Hematogenous spread of tubercle bacilli to any part of the body, particularly the apical-posterior portion of the lungs, epiphyses of the long bones, kidneys, vertebral bodies and meninges, may occur. In 95% of cases, after about 3 weeks of uninhibited growth of bacilli, the host immune system suppresses bacillary replication before symptoms or signs develop. Foci of infection in the lung or other sites resolve into epithelioid cell granulomas which may have caseous and necrotic centers. Tubercle bacilli can survive in this material for years and the host's resistance determines whether the infection ultimately resolves without treatment, remains dormant or becomes active. Foci may leave nodular scars in the apices of one or both lungs (Simon foci), calcified scars from the primary infection (Ghon foci) or calcified hilar lymph nodes (Grange, 2006; Martin and Lazarus, 2000).

Post primary TB may involve a non-respiratory site alone or in combination with respiratory disease. Post primary TB may occur by reactivation or reinfection. Reactivation means the dormant bacilli persisting in tissues after primary infection start to multiply and reinfection

means a repeat infection in a person who has previously had primary infection. Any organ initially seeded may be site of reactivation but occurs most often in the lung apices. The characteristic features of post primary TB include extensive lung destruction with cavitation, SS+, upper lobe involvement and usually no intrathoracic lymphadenopathy (Harries *et al.*, 2005).

3.7 Host defenses

The immune response against TB plays a fundamental role in the outcome of *M. tuberculosis* infection. *M. tuberculosis* stimulates both a humoral and a cell-mediated immunity. Although circulating antibodies appear, they do not convey resistance to the organism. Instead, the main component of the host defense against *M. tuberculosis* is a cell-mediated immune response (Harvey *et al.*, 2001). The tubercle bacilli reside inside a compartment within the macrophage and their antigens are presented by MHC class II molecules to CD4+ T lymphocytes (TH 1 subset). These cells play an important role in the protective response against *M. tuberculosis* and when they are absent, growth of the bacilli cannot be controlled (Pando *et al.*, 2007).

Cells of helper/inducer phenotype (CD4+) up-regulate population of antigen-specific effector (CD4+) and cytotoxic (CD8+) T cells. The main function of CD4+ T cell is the production of cytokines like interferon-gamma (IFN-γ), which activates macrophages and promotes bacilli destruction (Goldsby *et al.*, 2003). It also helps to develop CD8+ T cell mediated response. CD8+ T cells proved to be efficient in lysing infected cells and in reducing the number of intracellular bacteria. The mechanisms of control of the bacterial load seem to be associated with granular exocytosis involving perforin and granzymes. Granulysin, which is found in CD8+ T granules, is the molecule responsible for killing the bacterium. CD4+ T cells may also participate in the induction of apoptosis of infected cells and the subsequent reduction of bacterial viability through the CD95 Fas ligand system (Pando *et al.*, 2007).

3.8 Acid fast bacilli

M. tuberculosis is slender, straight or slightly curved rod with rounded ends, measuring 0.3-0.6 µm in width and 1-4 µm in length. 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 (mycolic **Chapter VIII: References**

52-62

acid, waxes and phosphatides) accounting up to 60% of the cell wall. Because of this distinctive property, it has its characteristic staining (Jolik *et al.*, 1992). *M. tuberculosis* is rarely pleomorphic, it does not elongate into filaments and does not branch in chains when observed in clinical specimens and culture

Mycobacteria are difficult to stain. The large amounts of lipid present on their cell wall makes them impermeable to the gram stain and appearance in a gram stained specimens 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 and the cell wall mycolic acid residues retain the primary stain even after exposure to acid-alcohol or mineral acids. A counter stain is just employed to highlight the stained organisms for easier microscopic recognition (Watt *et al.*, 1996).

There are several methods of determining the acid-fast nature of mycobacteria, such as hot staining method (ZN staining), cold staining methods (Kinyoun staining, Tam Tham Hok's method and modified Kinyoun staining) and flurochrome staining method. ZN staining and flurochrome staining are the two most widely used method to observe AFB.

3.9 Diagnostic approach for TB

Accurate and early diagnosis of TB is very important for its effective management. Several methods for the diagnosis of tuberculosis are available which include tuberculin test, radiological examination and other imaging methods, and SSM (Katoch, 2003). A complete medical evaluation must include medical history, physical examination, chest radiography and microbiological examination (CDC, 2007).

3.9.1 Medical history

The medical history includes obtaining the symptoms of PTB: productive, prolonged cough of three or more weeks, chest pain and hemoptysis. Systemic symptoms include fever, chills, night sweats, appetite loss, weight loss and easy fatigability (Harries *et al.*, 2005). Other parts of the medical history include prior TB exposure, infection or disease, past TB treatment, demographic risk factors for TB (e.g. country of origin, age, ethnic or racial group and occupation) that may increase the patient's risk for exposure to TB or to drug-resistant TB and medical conditions that increase risk for PTB disease, such as HIV infection (CDC, 2007; Harries *et al.*, 2005).

3.9.2 Physical examination

A physical examination is done to assess the patient's general health and to find other factors which may affect the TB treatment plan. It cannot be used to confirm or rule out PTB.

3.9.3 Chest radiography

A chest radiography is the usual step in evaluation of an individual with PTB symptoms. However, it is important to be aware that chest radiography has sustantial limitations for the diagnosis of PTB. The typical findings of chest radiography includes lesions mainly in apical posterior or superior segment of lungs (in 90% of cases), volume loss and cavitation. Atypial features includes hilar and medistinal lymphadenopathy, non-cavitary infiltrates and lower lobe involvement, seen particularly in patients with HIV infection or diabetes or renal failure (Menzies and Pourier, 2000). Diagnosis by means of a radiographic examination is unreliable, an abnormalities identified on a chest radiography may be due to PTB or due to a variety of other conditions. Thus, the abnormalities on chest radiography may be suggestive of but never diagnostic of PTB (Enarson *et al.*, 2000).

3.9.4 Tuberculin skin test

The tuberculin skin test is a major tool to diagnose PTB infection. In addition, this test is useful in an epidemiologic survey to define the prevalence of infection in population groups or to estimate prevalence or risk of infection in certain population groups. There are three types of tuberculin tests: Mantoux test, Heaf test and Multiple puncture tests (like Tine test)

(Menzies and Pourier, 2000; Perkins and Cunningham, 2007). The tuberculin skin test consists of the intradermal injection of a small amount of purified protein derived from *M. tuberculosis*. In a person who has previously been exposed and has developed cell-mediated immunity to these tuberculin antigens, a delayed cell-mediated reaction will occur within 48-72 hours. The reaction will cause localized swelling and manifest as induration of the skin at the injection site. In persons who are newly exposed and become infected with tubercle bacilli, this cell-mediated reaction to tuberculin will not be manifested immediately. It will develop between 3 and 8 weeks after the acquisition of infection. A positive tuberculin test does not mean the patients has active PTB and a negative test does not exclude PTB. Tuberculin screening of low risk population is generally discouraged, although testing may be performed for individuals (Menzies and Pourier, 2000).

3.9.5 Microbiological examination

The diagnosis of PTB can be firmly confirmed by demonstration of *M. tuberculosis* in the sputum. The two methods for establishing bacteriological diagnosis are finding of AFB in sputum smears and *M. tuberculosis* in culture. Laboratory diagnosis of PTB is to be assessed in relation to the sensitiveness of various diagnostic methods and its cost. In the developing countries, where the resources for the TB control are meagre, this sputum smear examination and culture become still more important in PTB diagnosis. The sputum smear examination provides the presumptive diagnosis of PTB whereas culture provides the definite diagnosis of PTB.

3.9.5.1 AFB microscopy

AFB microscopy has been used as in aid in TB diagnosis for many years. The presence of AFB on sputum smear indicates PTB disease. Two most widely used staining methods to observe AFB in sputum smears are carbol-fuschin (ZN) staining and fluorochrome staining. In carbol-fuchsin staining, AFB appears red against a blue or green background whereas in flurochrome staining, it appears as fluorescent rods, yellow to orange (the colour may vary with filter system used) against a paler yellow or orange background (Kantor *et al.*, 2007). In Nepal, ZN method is most widely used and flurochrome staining is used only in the central level.

3.9.5.2 Culture

The culture for *M. tuberculosis* is considered as the gold standard in diagnosis of TB. A positive culture for *M. tuberculosis* confirms the diagnosis of TB. A culture method is more sensitive and specific than AFB microscopy. It can find mycobacteria even when they are present in concentration of about 10-100 mycobacteria per ml of specimens (Glassroth, 1993; Grange, 1990). Despite its enhanced sensitivity as well as specificity, culture is of little clinical use, because of prohibitively slow, requiring 4-8 weeks before a positive culture for *M. tuberculosis* can be identified and requirement of well-equipped laboratory, which is not commonly available in many low-income and middle-income countries (Gebre *et al.*, 1995; Glassroth, 1993).

3.9.5.2.1 Culture media

Traditionally, culture has been grown on solid (egg-based and agar-based) and liquid media. The egg-based solid media are Lowenstein-Jensen (LJ) and Ogawa media. LJ media is the most commonly used media in the laboratories. The agar-based solid media are the various Middlebrook formulations (7H9, 7H10, and 7H11) media and the liquid media are Herman Kirchner and Dubos Oliec acid-Albumin media (Kantor *et al.*, 2007). Radiometric liquid culture such as BACTEC TB-460 system using a broth with radiolabelled C-palmitate as its sole carbon source has been also available for many years (Drobniewski *et al.*, 2003).
New fully automated systems that rely on non-radiometric detection of mycobcterial growth have been developed to culture *M. tuberculosis*. BACTEC 9000, Mycobacterial Growth Indicator Tube (MGIT), VersaTREK (previously known as ESP system II) and BacT/Alert 3D (previously known as MB/BacT) are some of them (Tortoli and Palomino, 2007). These techniques are more expensive than the conventional culture method.

Blood agar is an alternative culture medium for isolation of mycobacteria. Blood agar is at least as efficient as the widely recommended egg based media. *M. tuberculosis* grows within 1 to 2 weeks on blood agar plates and it has been reported that the average number of colonies isolated from clinical specimens on blood agar is significantly higher than the number of colonies on the egg-based medium. Blood agar can also be used as an alternative medium for

drug susceptibility testing of *M. tuberculosis*. It could be used for the culture of mycobacteria especially in resource-limited countries (Drancourt *et al.,* 2003; Waard and Robledo, 2007).

3.9.6 Serological diagnosis

A serological test for diagnosis of TB was first described in 1898 AD. The tuberculin skin test and more recently, the antigen-specific *ex vivo* induction of IFN-γ production have been used for the detection of infection with *M. tuberculosis*. IFN-γ assays that are now commercially available are Enzyme-linked immunospot (ELISPOT), T SPOT-TB assays, the original QuantiFERON-TB and its enhanced version QuantiFERON-TB Gold (Singh and Espitia, 2007). A wide variety of serological tests (TB Ig A EIA, PATHOZYME-MYCO IgG, IgA and IgM test) for the detection of antibodies in individuals suspected to have TB have also been evaluated to detect active disease (Chan *et al.,* 2000; Gennaro, 2000). The serological techniques are not useful in control program because of lack of sensitivity and specificity (Gebre *et al.,* 1995).

3.9.7 Nucleic acid amplification

Based on the newer knowledge about the specific gene sequences, several gene probes/gene amplification systems for TB have been developed. These molecular tools and methods can be used for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also molecular detection of drug resistance (Katoch, 2003). Nucleic acid amplification (NAA) assay is one of the most important technical advances for TB laboratory. NAA assays that are commercially available are Amplified Mycobacterium tuberculosis Direct Test (AMTD), Amplicor MTB Test, BD ProbeTec ET and Real-time PCR (Guillerm *et al.,* 2006; Tortoli and Palomino, 2007). Following the extraordinary development of the molecular methods, the identification of *Mycobacteria* spp, previously based on phenotypic investigations, suddenly started to rely on the genotypic methods. Some of the genotypic methods are PCR restriction-enzyme analysis (PRA) and DNA-probe technology like AccuProbe and Line probe assays (commercially available as INNO-LiPA Mycobacteria, GenoType Mycobacterium, GenoType MTBC) (Tortoli and Palomino, 2007).

In addition to conventional, automated and molecular diagnostic methods described, some new technologies have been proposed, such as phage-based assays (FAST-Plaque TB assay), rapid detection of growth by microscopic observation of microcolonies in solid or liquid media (Micro-colony method or thin-layer agar technique and MODS) and analysis of cell wall mycolic acids (TLC, GLC and HPLC) (Tortoli and Palomino, 2007).

Although NAA assay, genotypic methods and non conventional phenotypic methods are used worldwide, these methods are far from having revolutionized clinical mycobacteriology. Culture supported by microscopy still remains the gold standard and the molecular methods only represent a useful support in some cases, to speed up the diagnosis of TB. Hence, for the developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods like demonstration of AFB in smears is of great importance. No other diagnostic tool offers the affordability as well as efficiency in diagnosis of TB in public health setup, as SSM does (Shrestha *et al.,* 2006).

3.10 Sputum smear microscopy

The core diagnostic technology enshrined in current control strategies is SSM which was developed in the 1880's and has remained essentially unchanged since then (Murray *et al.,* 2003). SSM is one of the most effective and efficient tools for case-finding in NTP. It plays a very important role in the initial diagnosis of TB, monitoring of treatment and determination of eligibility for release from isolation (Peterson *et al.,* 1999). This method is rapid, specific and reasonably easy to perform. Early identification and isolation of PTB patients is of utmost importance in minimizing the risk of further epidemic spread. It is estimated that 60-70% of all TB cases are diagnosed by means of sputum smear examination (Githui *et al.,* 1993). In patients with active PTB, only an estimated 45% of infections are detected by SSM (Guillerm *et al.,* 2006).

SSM is an attractive technology for public health programs. It requires only one piece of equipment and can be used for more than one purpose, provides visual evidence of TB and in most instances is specific enough that no confirmatory testing is needed. However, only tiny amounts of material are examined, as little as 0.2 µl, even when viewing more than 100 microscopic fields, as a result bacilli must be present in high concentrations to be visible, typically over 10,000 AFB per ml of sputum (Perkins *et al.,* 2006). Though SSM is routinely described as a very simple test, it is highly dependent on the training and diligence of

microscopist, requires multiple examinations of smear and in programmatic conditions takes days rather than hours to complete, so that many patients drop out during the diagnostic process. The sensitivity of SSM in the diagnosis of PTB is far from perfect, although it has been reported to have greater than 80% sensitivity in some settings (Steingart *et al.,* 2006). Though the sensitivity of SSM is low, its specificity, simplicity and rapidity have made this procedure a popular method for early detection of active PTB (Saceanu *et al.,* 1993).

SSM clearly has advantages when it comes to speed and feasibility, and if sensitivity could be improved, it has the potential to become an even more valuable tool for NTP around the world. The performance of SSM can be significantly improved, if sputum is liquefied with one or other chemical reagent and then concentrated by centrifugation or sedimentation prior to acid-fast staining (Angeby *et al.,* 2004). Reports describing newer sputum processing methods as well as calls for re-examination of existing methods have prompted interested in the assessment of chemicals processing and sputum concentration to improve the sensitivity of SSM (Perkins, 2000; Trebucq, 2004). Chemicals, such as sodium hydroxide (NaOH) and a solution of N-acetyl L-cysteine and NaOH (NaLC-NaOH) for liquefaction of sputum together with concentration are widely used in modern laboratories. However, the methods aiming to concentrate bacilli by digestion of sputum with NaOCl and subsequent concentration by centrifugation or sedimentation have received much attention (Lawson *et al.,* 2006).
NaOCl was being advocated as a useful additive for SSM nearly 100 years ago (Ramsay *et al.*, 2006). It has the advantage of being available almost everywhere as household bleach. Moreover, it is an effective disinfectant which kills off *M. tuberculosis* and thus probably improves safety in the laboratories that lack adequate biosafety facilities. NaOCl may kill the bacilli by destroying cell wall or by reacting with thiol or amino group present in cytoplasm or cytoplasmic membrane (Denyer and Russell, 2005). NaOCl is also effective against HIV. Although most HIV particles present in sputum are non-infectious, it might be of at least psychological importance to laboratory staff, especially if the sputum is haemoptoic (Angeby *et al.,* 2004).

In a study, Uy *et al.,* (1988) evaluated the clorox concentration method for demonstration of AFB in sputum. A total of 297 sputum were collected from 101 patients with clinical and radiological evidence of PTB. The results showed that the positivity rate of the direct smears was 35.7%, that of clorox (4.5% NaOCl) was 45.8% and 46.1% for sputufluol (0.02% NaOCl). Statistical significance was noted between the direct method and two concentration

methods as to the recovery of AFB in the sputum ($P<0.001$). There was also a great degree of discordance between these methods (direct versus concentration). In addition, no statistical significance was noted when the two concentration methods were compared to each other ($P>0.05$). The study suggested that Clorox concentration method can be useful in the hospital to increase the yield of AFB in sputum smear and should replace the direct method in the epidemiological case-finding activities.

Gebre *et al.,* (1995) compared the microscopy of smears made directly from sputum with microscopy after liquefaction of sputum with NaOCl and concentration of mycobacteria by centrifugation, and reported that the use of NaOCl method increased the number of sputum sample positives for AFB by more than 100%. The sensitivity of smears for AFB compared with culture was 30.8% when smear were prepared directly from sputum and 69.2% when smears were prepared after NaOCl treatment and centrifugation. There was a more than 10 fold increase in average number of AFB seen per microscopic fields in smear prepared after NaOCl treatment compared to smears made directly from sputum. Specificity was 100% for both methods.

Miorner *et al.,* (1996) reported that out of 550 sputum, 91, 114, 115 sputum were positive for AFB by direct microscopy, NaOCl-centrifugation method and NaOCl-sedimentation method respectively, according to technician A. According to technician B 94, 112, 114 sputum were found positive for AFB by direct microscopy, NaOCl-centrifugation method and NaOCl-sedimentation method respectively. Statistically significant difference was noted between the results of direct microscopy and concentration methods. No statistical significance was noted between the results of two concentration methods. The study suggested that the sedimentation method could be an alternative to concentration of tubercle bacilli by centrifugation.

Wilkinson and Sturm (1997) reported that the correlation between the direct smears and NaOCl-centrifuged smears was high but the extra cases diagnosed after NaOCl-concentration method was offset by a same number that, initially positive, were negative after NaOCl-concentration method. Overall diagnostic sensitivity of direct microscopy was not increased by sputum liquefaction and concentration. The study suggested that the value of this technique may lie in combination with direct microscopy. The study also suggested that by limiting the specimen examination to one per patient and making an initial direct smear with subsequently concentration only if the direct smear was negative, the sensitivity of direct smears can be improved without any reduction in specificity.

In a study done by Habeenzu *et al.,* (1998), sputum samples were collected from 488 TB suspects and compared the results obtained by the NaOCl-centrifugation method with direct microscopy. The study reported that the NaOCl-concentration method was more sensitive than direct microscopy. The sensitivity was found 76.3% for NaOCl-centrifugation method and 43.3% for direct microscopy. The specificity was found to be 100% for both methods. Direct sputum smears stained with auramine-phenol were used as reference standard. The study suggested that the application of this method would increase the efficacy of TB control programs.

In a study conducted by Angeby *et al.,* (2000), out of 303 sputum, 39 were positive with the NaOCl method compared to 28 with the direct smear, leading to a significant increase in the number of positive samples after concentration of 39% ($P<0.05$). The sensitivity, specificity, positive and negative predictive values of NaOCl-centrifugation method were found to be 62%, 99%, 95% and 89% respectively and that of direct method were 47%, 99%, 97% and 86% respectively. In next studies, out of 971 sputum, 100 (10.3%) were positive using the NaOCl method while 75 (7.7%) were positive using the direct method. 27 slides were positive with the NaOCl method only while 2 were positive with the direct method only. The number of positive samples increased by 33% using the NaOCl method ($P<0.001$). In third studies, a total of 1422 sputum were processed and reported that 33 (2.3%) sputum turned out positive with the direct method while 37 (2.6%) were positive with the NaOCl method. The increase in the number of positive slides was 12%.

Bruchfeld *et al.,* (2000) also reported that overall sensitivity increased from 54.2% using conventional direct microscopy to 63.1% after NaOCl-concentration method ($P<0.0015$). In HIV-positive patients, overall sensitivity increased from 38.5% using conventional direct microscopy to 50.0% after NaOCl treatment and concentration ($P<0.0034$).

In a study done by Van Duen *et al.,* (2000), examination of 3287 sputum samples in duplicate disclosed that more positives were not found by NaOCl-sedimentation method. The positive percentage rose slightly from 15.5% for direct smears to 16.6% after NaOCl-sedimentation method using the American Thoracic Society threshold. When patients rather than individual smears were counted, more suspects were detected by NaOCl-sedimentation method (10% gain on average) but with considerable variation between the centres (range 6–16%).

Aung *et al.,* (2001) compared the microscopy of smears made directly from sputum with microscopy after liquefaction of sputum with NaOCl and concentration of mycobacteria by centrifugation, and reported that out of 948 sputum, 248 (26.2%) were positive for AFB by direct microscopy and 293 sputum (30.9%) were positive for AFB by the NaOCl method. There was a significant increase in the number of AFB positive samples by the NaOCl method ($P<0.05$).

In a study done by Saxena *et al.,* (2001), a total of 304 sputum specimens were studied by direct ZN staining and after NaOCl treatment and centrifugation. The use of NaOCl method increased the number of positive samples from 52 to 96. No false positives were detected by either method.

In a study done by Farnia *et al.,* (2002), a total of 430 sputum specimens were processed by NaOCl-sedimentation method and direct microscopy. The study reported that the number of AFB positive smears increased from 41 by direct microscopy to 63 by NaOCl-treated sputum. Indeed of 28 false negative reported by direct smear microscopy, 22 (78%) turned out to be positive by the NaOCl-sedimentation method. The sensitivity and specificity of NaOCl-sedimentation method were 78% and 96%, and that of direct microscopy were 46% and 90% respectively.

Yassin *et al.,* (2003) reported that sputum specimens treated by NaOCl-sedimentation method had 26% (52/200) positivity compared to 17.5% (35/200) of unbleached smears ($P<0.001$). The sensitivity, specificity, positive predictive values and negative predictive values of bleached smears were 92.3%, 93.4%, 78.3% and 97.7% respectively, against case definition.

In a study done by Gebre-Selassie (2003), the rate of recovery of AFB from sputum was 8.5%, 25.5% and 38.0% for direct smear microscopy, NaOCl-centrifugation method and NaOCl-sedimentation method respectively. Both the concentration method increased the yield of AFB by more than 3 fold compared with the direct microscopy of sputum ($P < 0.05$). The sensitivity of NaOCl-centrifugation method and NaOCl-sedimentation method were found to be 75% and 77.9% respectively, and the specificity to 100% for both techniques.

In a study conducted by Mutha *et al.,* (2005), 664 sputum specimens were collected from 297 chest symptomatic patients. The study reported that out of 297 chest symptomatics, 16 cases (5.38%) were positive by direct microscopy and 27 cases (9.09%) were positive by NaOCl-centrifugation method. The NaOCl method is safe, cheap, easy and sensitive. It can be applied for improved detection of *M. tuberculosis* in hospitals and laboratories, especially in settings where mycobacterial culture facilities are not available.

Lawson *et al.,* (2006) compared a NaOCl digested smears with direct SSM and culture. The study reported that 455 (60%) patients were culture positive. Of these, 235 (31%) had 'definite' PTB and 223 (29%) had 'very likely' PTB (SS- and culture positive). WHO case definition identified 51% (235/458) of the patients with 'definite' or 'very likely' PTB. One digested smear detected 219 (93%) of the 235 patients with 'definite' PTB and 10 patients with 'very likely' to have PTB (sensitivity [95%CI] 50%, specificity 99% [97–100%]). The positive and negative predictive values for one digested smear were 98% (95–99%) and 56% (52–60%) respectively, which were not different (P<0.5) to the WHO case definition (100% and 57% respectively).

3.10.1 Limitation of sputum smear microscopy

Although relatively rapid, simple and inexpensive, SSM suffers from a major drawback of having low sensitivity. This disadvantage of SSM 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 PTB by direct sputum examination ranges from 60-70%. The isolation of mycobacteria 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 SSM which include the availability of saliva instead of sputum, as well as quality and quantity of sputum. A large laboratory work load may also influence the sensitivity. False negative results 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 programs. It requires equipment

that is difficult to maintain in field settings, yield results depend upon the studious attention of the trained and motivated laboratory technician, and it is notoriously insensitive especially in control programs. Besides this, the smear examination requires sputum collection, smearing, drying, staining and the need of duplicate or triplicate sputum examination further compounds the problem (Perkins, 2000).

WHO recommended that negative results should only be reported after the examination of at least 300 microscopic oil immersion fields or equivalent fluorescent view fields. Therefore, when SSM is performed correctly, it can be time consuming and laborious (Toman, 1997). Negative results and slow reporting ultimately may erode patient faith in the services of the laboratory and health system. Again, a negative smear result does not exclude the diagnosis of TB, 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 sputum with positive smear failed to grow in culture while 30% are smear negative but culture positive (Barez *et al.,* 1995). Many data suggested that the SSM is a poor screening technique in a population where the prevalence of TB is low and where the burden of HIV and AIDS is high (Boyd and Marr, 1975). The probability of detecting AFB in sputum is reduced in HIV positive cases of PTB.

3.10.2 Factors influencing sensitivity and specificity of AFB smears

3.10.2.1 False positive results

The main reason behind the false positive results is acid-fast particles other than tubercle bacilli which include certain food particles (e.g. waxes, oils), other microorganisms (e.g. Environmental AFB, Non-tuberculosis and *Nocardia* species), precipitated stains, artifacts, inorganic materials (e.g. fibres, pollens), scratches on the slide which retain the red stain and accidental transfer of AFB from positive slide to negative one (Harries *et al.,* 2005; Toman, 1997).

3.10.2.2 False negative results

False negative results may be due to poor quality of the sputum specimens, improper sputum collection, improper storage of sputum specimens and stained smears, failure to select suitable sputum particles for smear preparation, improper preparation of smears, improper staining of slides and improper examination of smears. The other reasons for the false negative results may be administrative errors (like misidentification of patients, confusion of code numbers of specimens and slides, false recording or reporting and mistakes in labeling containers) and reading errors (Harries *et al.,* 2005; Toman, 1997).

CHAPTER-IV

4. MATERIALS AND METHODS

The present study was conducted at the high safety laboratory of NTC, Thimi, Bhaktapur, Nepal from May 1st, 2007 to November 15th, 2007. Routine new suspected PTB patients, belonging to different region of Nepal, who visited NTC were only included in this study. A random sampling was done and 159 clinically suspected patients were chosen for study.

4.1 Materials

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

4.2 Methods

4.2.1 Sample collection

Three sputum from each patient, one 'on-spot' and two early morning sputum were collected on the consecutive days. Patients were provided with rigid, clean, leak proof, disposable, semi-transparent, wide-mouthed, screw-capped and easily-labeled plastic container. Patients were instructed to provide about 5ml deep-cough sputum, not saliva or nasal secretions.

4.2.2 Laboratory examination of sputum

4.2.2.1 Study method

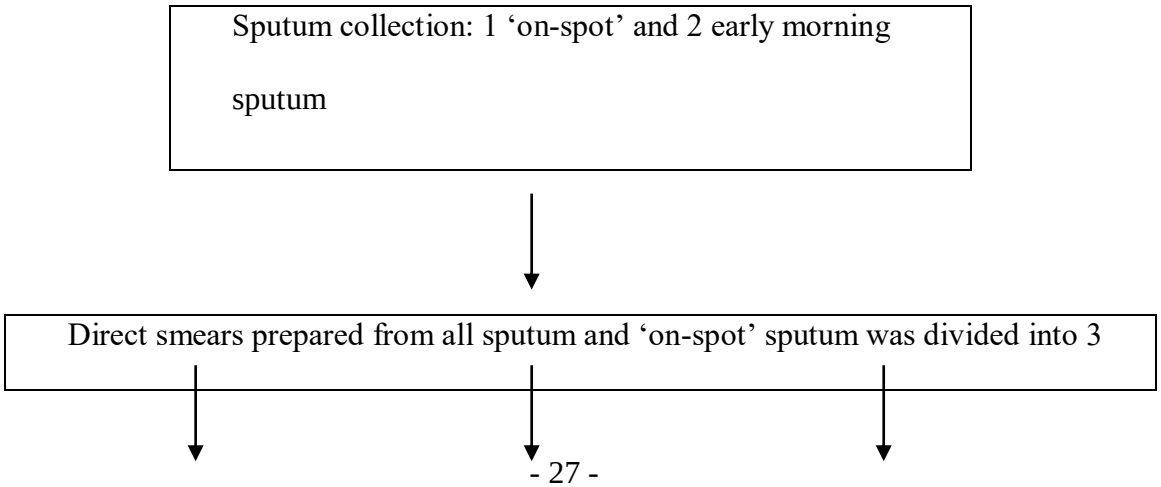
Direct smear of each sputum specimen was prepared and stained by ZN staining. 'On-spot' sputum was divided equally into three parts in 20ml screw-capped plastic centrifuge tubes. The tubes were labeled A, B and C respectively; tube A was used for NaOCl-centrifugation method, tube B for NaOCl-sedimentation method and tube C for culture. After treatment of sputum by NaOCl-centrifugation and NaOCl-sedimentation methods, digested smears were prepared and stained by ZN staining. NaOCl-centrifuged and NaOCl-sedimented 'on-spot'

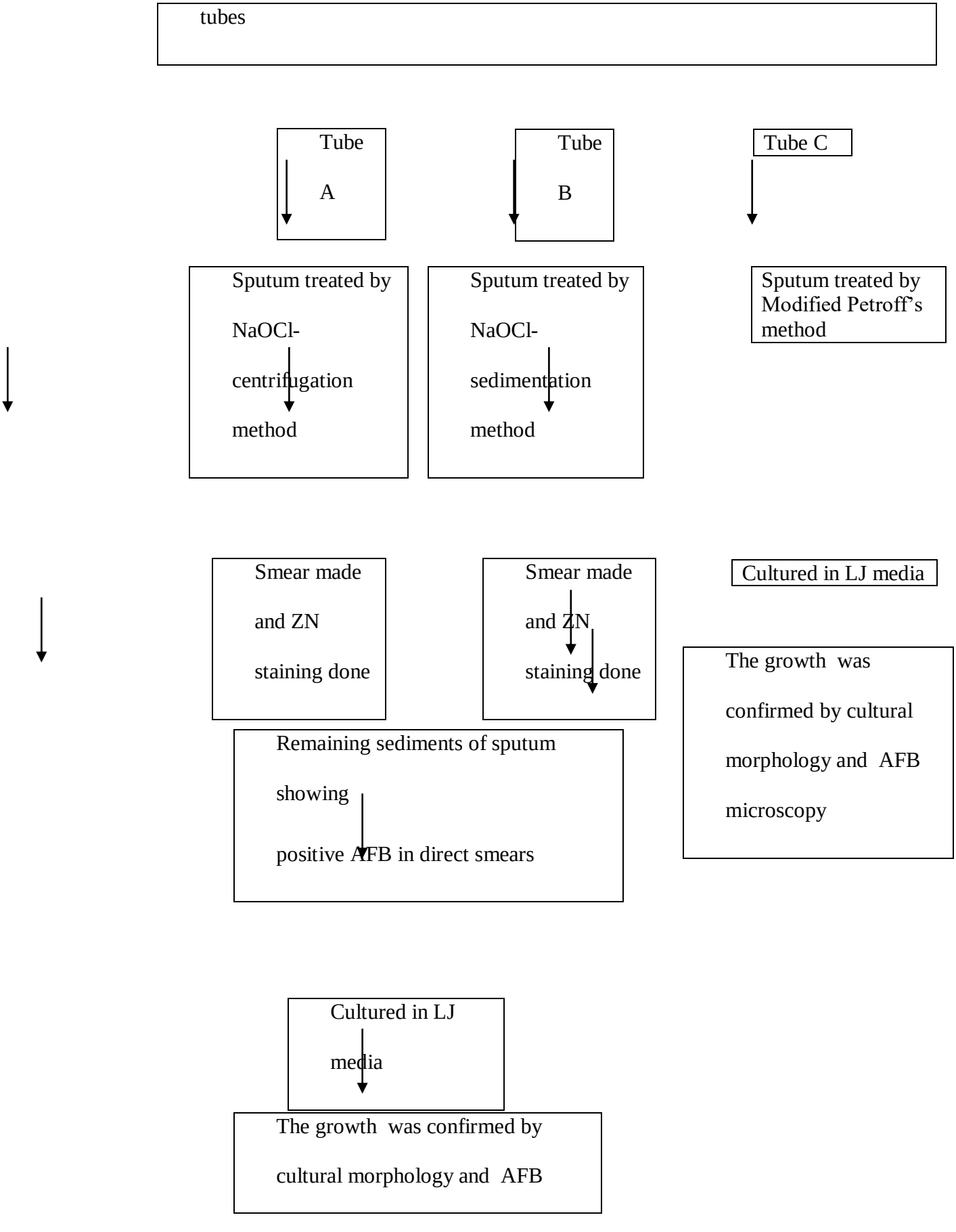
sputum showing positive AFB in direct ZN smears were also used for culture to ascertain the sterilizing activity of NaOCl.

With culture employed as the gold standard, it was compared with direct 'on-spot' sputum ZN smears, NaOCl-centrifugated ZN smears, NaOCl-sedimented ZN smears and the standard three smears strategy. Diagnosis based on a single NaOCl-digested smear i.e. a single NaOCl-centrifugated ZN smear and a single NaOCl-sedimented ZN smear were compared with WHO case definition.

In recording and reporting of microscopic results, the following reporting scale was used for ZN staining as per the guidelines given by IUATLD/WHO (Kantor et al., 2007).

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+




tubes
Tube A
Tube B
Tube C
Sputum treated by NaOCl-centrifugation method
Sputum treated by NaOCl-sedimentation method
Sputum treated by Modified Petroff's method
Smear made and ZN staining done
Smear made and ZN staining done
Cultured in LJ media
The growth was confirmed by cultural morphology and AFB microscopy
Remaining sediments of sputum showing positive AFB in direct smears
Cultured in LJ media
The growth was confirmed by cultural morphology and AFB

microscopy

Fig 1: Flow chart of sample processing

4.2.2.2 Microscopic examination of sputum smears

4.2.2.2.1 Preparation of smear

1. A slide was cleaned with blotting paper and labeled at one end.
2. A small proportion of a purulent part of sputum or NaOCl digested and concentrated sediment was smeared evenly with an uneven end of broom stick over an area of approximately 2×3 cm.
3. The smear was air dried and heat 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.

4.2.2.2.2 NaOCl treatment method

4.2.2.2.2.1 NaOCl-centrifugation method

1. Equal volume of sputum and NaOCl was mixed.
2. The mixture was left for 15 minutes and shaken by hand at regular interval of time.
3. 8 ml of distilled water was added to it.
4. The mixture was centrifuged at 3000 rpm for 15 minutes.
5. Supernatant was removed and sediment was dissolved in remaining supernatant.
6. A drop of re-suspended sediment was transferred to slide with the help of pasteur pipette to make a smear.

4.2.2.2.2.2 NaOCl-sedimentation method

1. Equal volume of sputum and NaOCl was mixed.
2. The mixture was left for 15 minutes and shaken by hand at regular interval of time.

3. 8 ml of distilled water was added to it.
4. The mixture was left for overnight (15-18 hours) at room temperature.
5. Supernatant was removed and sediment was dissolved in remaining supernatant.
6. A drop of re-suspended sediment was transferred to slide with the help of pasteur pipette to make a smear.

4.2.2.2.3 Ziehl-Neelsen staining method

1. 0.3% 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.
5. The slides were decolourised with 20% sulphuric acid for 3 minutes.
6. The slides were rinsed thoroughly with tap water and excess water was drained off.
7. The slides were flooded with 0.1% methylene blue 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 and were allowed to air dry.
9. The slides were examined under microscope in 1000x oil immersion.

4.2.2.3 Sputum culture

4.2.2.3.1 Modified Petroff's method

1. Equal volume sputum and 4% NaOH was mixed vigorously and was let to stand for 15 minutes.
2. The mixture was centrifuged at 3000 rpm for 15 minutes.
3. The supernatant was discarded and 15 ml of distilled water was added to re-suspend the sediment.
4. The mixture was again centrifuged at 3000 rpm for 15 minutes.
5. Supernatant was decanted. The sediment was re-suspended in the remaining supernatant.
6. The re-suspended sediment was used for inoculation.

4.2.2.3.2 Inoculation and incubation

1. 1-2 drops of centrifuged sediment were inoculated into two LJ slope per specimen by pasteur pipette.
2. It was distributed over the surface of LJ media.
3. The tubes were incubated at 37°C for 24 hours in a slanted position with loosen caps.
4. The caps were tightened and incubated at 37°C at upright position until growth was observed.

4.2.2.3.3 Culture examination schedule

1. Culture tubes were examined after 48-72 hours of incubation to detect any contamination.
2. The tubes were then examined on 7th day for rapidly growing mycobacteria.
3. The tubes were examined once weekly thereafter, up to 8 weeks for slow growers including *M. tuberculosis*.

4.2.2.3.4 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). The growth of *M. tuberculosis* was confirmed by a typical colony morphology, appearance of granular suspension when emulsified in saline or water used for making smears and microscopy for AFB. Cultures that showed no growth after 8 weeks were regarded as 'negative' and were discarded.

4.2.2.4 Biocidal activity of NaOCl

1. 1-2 drops of NaOCl-centrifuged and NaOCl-sedimented re-suspended sediment were inoculated into two LJ slopes with the help of pasteur pipette.
2. It was distributed over the surface of LJ media.
3. The tubes were incubated at 37°C for 24 hours in a slanted position with loosen caps.
4. The caps were tightened and incubated at 37°C at upright position.
5. The tubes were observed for the growth of *M. tuberculosis*.

4.3 Reporting of TB cases

A patient was considered as a ‘definite case’ if two or more positive direct smears for AFB or one positive direct smear with one culture positive for *M. tuberculosis* and as a ‘unlikely’ to have PTB, if none of the three direct smears were positive for AFB and no growth in culture. Patients with negative SSM but positive culture were considered to be ‘very likely’ to have PTB but would be considered to be SS-. Patients with only one positive direct smear but negative culture were considered to be ‘less likely’ to have PTB.

4.4 Statistical analysis

All the data entry, cross tabulation and descriptive analysis were done by using SPSS version 11.5. McNemar’s $\chi 2$ test was used for dichotomous data and the kappa statistics was used to assess the agreement of tests. The sample size was calculated by using Winpepi version 1.67.

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

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

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

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

CHAPTER-V

5. RESULT

A total of 477 direct smears, 159 NaOCl-centrifuged smears, 159 NaOCl-sedimented smears and 191 LJ cultures were prepared from 159 suspected PTB patients. 37 (23.2%) patients were found to be culture positive and 116 (73%) patients were culture negative. 6 (3.8%) LJ cultures were found to be contaminated. No growth was observed in the LJ media inoculated with NaOCl-treated sputum showing positive direct ZN smear for AFB.

Table 1: Age wise and sex wise distribution of definite PTB cases in study group

Age and Sex		Number (n)	definite PTB	
			Positive % (n)	Negative % (n)
Age	Less than 15 years	2	0 (0)	100 (2)
	15-24 years	44	20.4 (9)	79.6 (35)
	25-34 years	32	15.6 (5)	84.4 (27)
	35-44 years	37	13.6 (5)	86.4 (32)
	45-54 years	23	17.3 (4)	82.7 (19)
	55-64 years	10	20 (2)	80 (8)
	More than 64 years	5	20 (1)	80 (4)
Sex	Male	124	17.7 (22)	85.4 (107)
	Female	29	13.7 (4)	72.4 (21)
Total		153	16.9 (26)	83.1 (127)

Among 153 suspects, 17.7% (n=22) male and 13.7% (n=4) female were diagnosed as definite PTB cases respectively. Maximum numbers of definite PTB cases (20.4%) were observed in the age group of 15-24 years (Table 1).

The validities of the direct 'on-spot' sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy using culture as the gold standard for PTB diagnosis were shown in Table 2, Table 3, Table 4, and Table 5.

Table 2: Comparison of results obtained with 'on-spot' sputum smears stained by ZN staining directly and culture

Direct ZN smears	Culture			Sensitivity %	Specificity %	Predictive value	
	Positive	Negative	Total			Positive%	Nega
Positive	13	3	16				
Negative	24	113	137	35.1	97.4	81.2	82
Total	37	116	153				

The total positive results obtained by direct 'on-spot' sputum ZN smears was 16, out of which the true positive (TP) was found to be 13 (81.2%) and false positive (FP) was found 3 in number (18.7%). This method detects 137 as total negative, out of which 113 (82.5%) were true negative (TN) and 24 (17.5%) of them were false negative (FN) (Table 2).

Table 3: Comparison of results obtained with 'on-spot' sputum smears stained by ZN staining after NaOCl-centrifugation method and in culture

NaOCl-centrifug-ed ZN smears	Culture			Sensitivity %	Specificity %	Predictive value	
	Positive	Negative	Total			Positive %	Negati %
Positive	25	3	28				
Negative	12	113	125	67.5	97.4	89.2	90.4
Total	37	116	153				

The total positive results obtained by NaOCl-centrifuged ZN smears was 28, out of which the TP was found to be 25 (89.2%) and FP was found 3 in number (10.8%). This method detects 125 as total negative, out of which 113 (90.4%) were TN and 12 (9.6%) of them were FN (Table 3).

Table 4: Comparison of results obtained with 'on-spot' sputum smears stained by ZN staining after NaOCl-sedimentation method and in culture

NaOCl-sedimented ZN smears	Culture			Sensitivity %	Specificity %	Predictive value	
	Positive	Negative	Total			Positive %	Nega %
Positive	23	3	26	62.1	97.4	88.4	89
Negative	14	113	127				
Total	37	116	153				

The total positive results obtained by NaOCl-sedimented ZN smears was 26, out of which the TP was found to be 23 (88.4%) and FP was found 3 in number (11.6%). This method detects 127 as total negative, out of which 113 (89%) were TN and 14 (11.0%) of them were FN (Table 4).

Table 5: Comparison of results obtained with standard three smears strategy and in culture

Standard three smear strategy	Culture			Sensitivity %	Specificity %	Predictive value		V
	Positive	Negative	Total			Positive %	Negative %	
Positive	20	3	23	54	97.4	87	87	<
Negative	17	113	130					
Total	37	116	153					

The total positive results obtained by standard three smears strategy was 23, out of which the TP was found to be 20 (87%) and FP was found 3 in number (13%). This method detects 130 as total negative, out of which 113 (87%) were TN and 17 (13%) of them were FN (Table 5)

In this study, 159 sputum were examined in parallel. Comparison of results obtained with 'on-spot' sputum smears stained by ZN staining directly and after NaOCl-centrifugation method and NaOCl-sedimentation method, and WHO case definition were shown in table 6, table 7, table 8, table 9 and table 10.

Table 6: Smear positivity among the 'on-spot' sputum stained directly by ZN staining and after NaOCl-centrifugation method

NaOCl-centrifuged ZN smears	Direct ZN smears					Total	Kappa value
	0	Scanty	1+	2+	3+		
0	131	0	0	0	0	131	0.53
Scanty	6	0	0	0	0	6	
1+	4	1	3	1	0	9	
2+	2	0	4	3	0	9	
3+	0	0	0	1	3	4	
Total	143	1	7	5	3	159	

The total yield of positive results by the NaOCl-centrifuged ZN smears was 28 (17.7%) compared to 16 (10.1%) by direct 'on-spot' sputum ZN smears. The kappa test for agreement between the two methods was 0.53 (Table 6).

Table 7: **Smear positivity among the 'on-spot' sputum stained directly by ZN staining and after NaOCl-sedimentation method**

NaOCl-	Direct smears	Total	Kappa

sedimented smears	0	Scanty	1+	2+	3+		value
0	133	0	0	0	0	133	0.58
Scanty	4	0	0	0	0	4	
1+	5	1	3	1	0	10	
2+	1	0	4	4	0	9	
3+	0	0	0	0	3	3	
Total	143	1	7	5	3	159	

The total yield of positive results was higher by NaOCl-sedimented ZN smears: 26 (16.3%) positive as opposed to 16 positive by direct 'on-spot' sputum ZN smears (10.1%) (Table 7).

Table 8: **Smear positivity among the 'on-spot' sputum stained by ZN staining after NaOCl-centrifugation method and after NaOCl-sedimentation method**

NaOCl-centrifuged smears	NaOCl-sedimented smears					Total	Kappa value
	0	Scanty	1+	2+	3+		
0	131	0	0	0	0	131	0.91
Scanty	2	4	0	0	0	6	
1+	0	4	9	0	0	9	
2+	0	0	1	8	0	9	
3+	0	0	0	1	3	4	
Total	133	4	10	9	3	159	

The total positive result yielded by NaOCl-centrifugation method was 28 (18.4%) as opposed to 26 positive by NaOCl-sedimentation method (16.9%) (Table 8).

Table 9: Smear positivity by NaOCl-centrifuged ZN smears compared with WHO case definition

Certainity of TB	number (N)	NaOCl-centrifuged ZN smears					
		0	Scanty	1+	2+	3+	Total Positive
Definite	26	3	4	8	7	4	23 (88.4%)
Very likely	14	9	2	1	2	0	5 (35.8%)
Less likely	0	0	0	0	0	0	0 (0%)
Unlikely	113	113	0	0	0	0	0 (0%)
Total	153	125	6	9	9	4	28 (70%)

NaOCl-centrifuged ZN smears detect 23 (88.4%) patients to have smear positive out of 26 definite cases. Similarly, it found 5 (35.8%) cases to have smear positive among 14 ‘very likely’ to have PTB cases. No smear positive case was found among ‘less likely’ and ‘unlikely’ to have PTB cases by this method. Thus, NaOCl-centrifuged ZN smears alone identified 70% (28/40) of the total cases with PTB (Table 9).

Table 10: Smear positivity by NaOCl-sedimented ZN smears compared with WHO case definition

Certainity of TB	number (N)	NaOCl-sedimented ZN smears					
		0	Scanty	1+	2+	3+	Total Positive
Definite	26	4	3	8	8	3	22 (84.7%)
Very	14	10	2	1	1	0	

likely	0	0	0	0	0	0	4 (28.6%)
Less likely	113	113	0	0	0	0	0 (0%)
Unlikely	153	127	5	9	9	3	0 (0%)
Total							26 (65%)

NaOCl-sedimented ZN smears detect 22 (84.7%) patients to have smear positive out of 26 ‘definite’ cases. Similarly, it found 4 (28.6%) cases to have smear positive among 14 ‘very likely’ to have PTB cases. No smear positive case was found among ‘less likely’ and ‘unlikely’ to have PTB cases by this method. Thus, NaOCl-sedimented ZN smears alone identified 65% (26/40) of the total cases with PTB (Table 10).

CHAPTER - VI

6. DISCUSSION AND CONCLUSION

6. 1 Discussion

Identifying SS+ PTB cases is a core component of TB control programs. Subsequent to abnormal radiological findings, tuberculin test and clinical symptoms, positive acid-fast smears from a patient's sputum provide a presumptive diagnosis of active PTB. Although attention has turned to NAA technology like PCR and related techniques which require more sophisticated laboratory methods and equipments, SSM remains the method of choice and currently the only microbiological method for confirmation of PTB applicable to control program in resource poor countries (Heifets and Levy, 1999). The probability of detecting bacilli by direct SSM increases with increasing density of bacilli, this probability appears to be in the neighborhood of 60% with 1000 bacilli and 95% with 10,000 per ml of sputum. Even with the poor microscopy, specimens containing 30,000 to 60,000 bacilli per ml will not usually be missed (Rieder *et al.,* 2007). SSM is a good test for identifying the most infectious cases, it is not, however, a sensitive test for TB diagnosis as compared to culture (Yassin *et al.,* 2003). The advantages of SSM are well known; it is inexpensive to perform, is very specific in high prevalence settings and detects the most infectious subset of patients. For these reasons, SSM rightly retains its primary role in case detection. However, technical shortcomings of SSM seriously limit both the extent and quality of its application, and ultimately its impact on TB control (Perkins *et al.,* 2006).

Clinical management of PTB cases in developing countries is hampered by the lack of a simple and effective diagnostic test. Correct diagnosis of PTB is needed to improve treatment, reduce transmission and control development of drug resistance. In patients with active PTB, only an estimated 45% of infections are detected by SSM (Guillerm *et al.,* 2006). This test, first developed in the 1880s and basically unchanged today, has the advantage of being simple, but is hampered by very low sensitivity. SSM may only detect half of all cases with active infection. Furthermore, a staggering 3 million people who present annually with

suspected PTB may not be properly diagnosed because their infection (so-called SS- PTB disease and potential source of future SS+ PTB cases) cannot be detected by SSM (Guillerm *et al.,* 2006; Onyebujoh *et al.,* 2006). So, there is need to standardize the SSM. This study evaluates NaOCl digestion of sputum to improve the sensitivity of SSM which is obviously crucial to NTP and its case-finding potential.

In the present study, among 153 suspected PTB patients, 16.9% patients were found to have definite PTB cases. Maximum number of definite PTB cases was observed in the age group of 15-24 years (20.4%). Considering the gender in definite PTB cases, more males were found to be suffering from the disease than females. 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.

Handling sputum specimens containing AFB poses a risk of infection to laboratory workers. The use of NaOCl digestion method of sputum may make laboratory workers feel safe from laboratory-acquired infection even if direct smear preparation may not pose a great risk of infection (Reid, 1957). It is well known that NaOCl effectively kills *M. tuberculosis*, which was also confirmed by this study. When the NaOCl-centrifuged and NaOCl-sedimented sputum was inoculated in LJ media, no growth of *M. tuberculosis* was seen in media. This result confirmed the biocidal activity of NaOCl. This disinfectant potential will increase biosafety in laboratories and may be even more important in settings with a high incidence of HIV, where a high proportion of laboratory staff could be HIV-infected and thus be more susceptible to PTB (Angeby *et al.,* 2004).

In the present study, the validity of direct 'on-spot' sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy were calculated by using culture as gold standard for diagnosis of PTB. The sensitivity of these methods were found to be 35.1%, 67.5%, 62.1% and 54% for direct 'on-spot' sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy respectively. The increased sensitivity of NaOCl method is attributed to greater concentration of AFB and to the fact that NaOCl removes debris and leaves clearer microscopic field for easy examination (Angeby *et al.,* 2000; Farnia *et al.,* 2002; Miorner *et al.,* 1996). However, smears prepared from NaOCl treated sputum were thin and not easily

visible to naked eye, extra care was required in labeling and staining the correct side of the slides.

In the present study, NaOCl-centrifuged ZN smears yielded 17.7% positive smears compared to 16.3% positive smears by NaOCl-sedimented ZN smears, 10.1% positive smears by direct 'on-spot' sputum ZN smears and 14.5% by standard three smears strategy. This suggested that the NaOCl-centrifuged ZN smears resulted in an increase in the smear positivity rate compared to the result yielded by the standard smears of the same specimen and standard three smears strategy. NaOCl-sedimented ZN smears also resulted in an increase in the smear positivity rate compared to the yield from the standard smears of the same specimen and standard three smears strategy. This data proves that the NaOCl-digested smear could be used instead of the standard smears. Although there was no statistically significant difference noted between the number of AFB positive sputum samples obtained by NaOCl-sedimented ZN smears and the number achieved by NaOCl-centrifuged ZN smear, the average numbers of AFB per microscopic field indicate that centrifugation method is more efficient for concentration of tubercle bacilli. All of the 'on-spot' sputum ZN smears positive on direct microscopy were also positive by both NaOCl-treated ZN smears, which support the validity of the NaOCl-digested smears. All of the NaOCl-sedimented ZN smears that were positive, were also found to be positive by NaOCl-centrifuged ZN smears. In addition, NaOCl-centrifuged ZN smears identified 2 positive smears that were negative by NaOCl-sedimented ZN smears indicating that NaOCl-centrifuged ZN smears is superior to other two methods.

26 'definite' PTB cases, 14 'very likely' to have PTB cases and 113 'unlikely' to have PTB cases were identified by WHO case definition. NaOCl-centrifuged ZN smears detect 23 (88.4%) patients to have smear positive out of 26 definite cases. It also found 5 (35.8%) cases to have smear positive among 14 'very likely' to have PTB cases and no smear positive case was found among 'less likely' and 'unlikely' to have PTB cases. Similarly, NaOCl-sedimented ZN smears detect 22 (84.7%) patients to have smear positive out of 26 'definite' PTB cases, 4 (28.6%) cases to have smear positive among 14 'very likely' to have PTB cases and no smear positive case was found among 'less likely' and 'unlikely' to have PTB cases. A single NaOCl-centrifuged ZN smear and single NaOCl-sedimented ZN smear thus identified 70% and 65% of PTB cases respectively. This proved that the NaOCl digestion method had potential to be used in routine laboratory diagnosis of PTB. NaOCl-centrifuged ZN smear had higher sensitivity i.e. 67.5% compared to 62.1% of NaOCl-sedimented ZN

smear, 35.1% of single direct ZN smear and 54% of standard three smears strategy. Although the sensitivity of NaOCl-treated ZN smears was high, NaOCl-centrifuged ZN smears missed 3 of 26 ‘definite’ PTB cases and NaOCl-sedimented ZN smears missed 4 of 26 ‘definite’ PTB cases. So, further studies should investigate whether this can be improved by using two or more NaOCl-treated smears or a combination of NaOCl and standard techniques.

The specificity of direct ‘on-spot’ sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy was found to be same i.e. 97.4%. This result suggested that AFB microscopy has high specificity. However, the specificity of smear examination methods should be interpreted with caution because it does not allow the differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT).

In the present study, the PPV and NPV were high for all the methods. The PPV and NPV for direct ‘on-spot’ sputum ZN smears, NaOCl-centrifuged ZN smears and NaOCl-sedimented ZN smears were found to be 81.2%/82.3%, 89.2%/90.4% and 88.4%/89% respectively, and that of standard three smears strategy was 87%/87%. These data suggested that all the methods had sufficient validity to predict the presence or absence of a disease in TB prevalence population.

In this study, the false positive rate was found to 18.7%, 10.8%, 11.6% and 13% for direct ‘on-spot’ sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy respectively. These data suggested that culture is the only definitive diagnosis of PTB that depends on isolation and identification of *M. tuberculosis*. Culture remained the gold standard diagnostic method for PTB. Culture methods are highly sensitive and specific than microscopy for detection of bacilli, since approximately 10-100 mycobacteria per ml of sputum is required for positive result while approximately 10^4 organism per ml of sputum is required to be seen by microscopic examination. 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.

The results of this study show that digestion of sputum with NaOCl followed by concentration of bacilli through centrifugation or sedimentation significantly increases the sensitivity of direct microscopy. In laboratories where centrifuge is not available, NaOCl-sedimentation method could be an alternative method. The method is easy to perform and only marginally more time consuming than direct microscopy, and it offers increased sensitivity and safety. Moreover, it reduces the time needed for examination of the slides by making the slides easier and faster to read.

The advantages of using a single NaOCl-digested smear in place of the conventional standard three direct smears on sputum collected over two days includes reducing the drop-out rate during the diagnostic process, easier and safer preparation of smears, and digested sputum is easier to manipulate since it is sterilized by the NaOCl treatment. In addition, it has the potential of a same day diagnosis when NaOCl-centrifugation method is applied. This approach also reduces the laboratory load and costs as fewer specimens need to be processed resulting in a reduction in time spent on clerking specimens and writing reports, reduction in smears being fixed, stained and examined, and the clearer background to the microscopic field may be expected to make the screening of negative smears faster with more time dedicated to each reading, enhancing the quality of reading of slides (Lawson *et al.*, 2006).

Considering the multifaceted advantages of NaOCl digestion, a single NaOCl-digested smear from the 'on-spot' sputum may be an appropriate approach for screening PTB suspects in resource poor countries. Suspects found to have SS+ PTB could start their anti-tuberculosis treatment within one hospital/health centre visit. From the patient's perspective, lost time will be saved (Yassin *et al.*, 2003).

This study provides evidence to support implementation of a NaOCl digestion method for diagnosing new cases of PTB especially in resource-poor countries. Its positivity rate, sensitivity and specificity were as good as standard three smears strategy. This method has the potential to improve over-burden services in developing countries like Nepal. However, it must be recognized that if safety cabinets are not available, the bleach should be mixed with sputum in container in which it has been deposited and one should not pour potentially infected sputum into a tube outside a safety cabinet.

It may be that this approach is what is needed to make SSM safe, simple and user-friendly to patients. However, introduction of this method does not limit the need of good training, supervision and careful quality control of all laboratory procedures.

6.2 Conclusion

5% NaOCl microscopy method significantly increased the sensitivity of direct microscopy and improved the laboratory safety. The method is simple and the only extra reagent is required i.e. NaOCl which is also readily available as household bleach. The cost of NaOCl digestion to current cost of SSM is likely to be negligible. The immediate application of this method in laboratories is feasible and could make a positive impact on the effectiveness of national TB control programs.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

TB has claimed its victims throughout the world. The highest priority for TB control is the identification and cure of infectious cases i.e. SS+ PTB cases. Sputum smear examination is still the mainstay in PTB diagnosis in the developing world. The present study was conducted in NTC, Thimi, Bhaktapur with the aim to evaluate safety and efficacy of NaOCl digestion methods of sputum for the laboratory diagnosis of PTB. In the present study, culture was employed as a gold standard method. The sample size in this study was 159 clinically suspected patients.

The major findings of the study are summarized as follows:

- Out of 153 PTB suspects, 81.1% (124) were males and 18.9% (29) were females among whom 17.7% (n=22) male and 13.7% (n=4) female were diagnosed as definite (SS+) PTB cases.
- Maximum numbers of definite (SS+) PTB cases were observed in the age group of 15-24 years (20.4%).
- 37 (23.2%) were found positive by culture. The total positivity yielded by direct 'on-spot' sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy were 10.1%, 17.7%, 16.3% and 14.5% respectively.
- The sensitivity of direct 'on-spot' sputum ZN smears, NaOCl-centrifuged ZN smears, NaOCl-sedimented ZN smears and standard three smears strategy were 35.1%, 67.5%, 62.1% and 54% respectively.
- The specificity was found to be same i.e. 97.4% in all methods
- The positive predictive value were found to be 81.2% in direct 'on-spot' sputum ZN smears, 89.2% in NaOCl-centrifuged ZN smears, 88.4% in NaOCl-sedimented ZN smears and 87% in standard three smears strategy.

- The negative predictive value were found to be 82.3% in direct 'on-spot' sputum ZN smears, 90.4% in NaOCl-centrifuged ZN smears, 89% in NaOCl-sedimented ZN smears and 87% in standard three smears strategy.

- When the direct 'on-spot' sputum ZN smears was correlated with NaOCl-centrifuged ZN smears, the kappa test for agreement was found to be 0.53.

- When the direct 'on-spot' sputum ZN smears was correlated with NaOCl-sedimented ZN smears, the kappa test for agreement was found to be 0.58.

- When NaOCl-centrifuged ZN smears was correlated with NaOCl-sedimented ZN smears, the kappa test for agreement was found to be 0.91.

- WHO case definition identified 26 'definite' PTB cases, 14 'very likely' to have PTB cases, 113 'unlikely' to have PTB cases.

- NaOCl-centrifuged ZN smears detect 88.4% and 35.8% cases to have smear positive out of 26 'definite' PTB cases and 14 'very likely' to have PTB cases respectively.

- NaOCl-sedimented ZN smears detect 84.7% and 28.6% cases to have smear positive out of 26 'definite' PTB cases and 14 'very likely' to have PTB cases respectively

- NaOCl-centrifuged ZN smears alone identified 70% of PTB cases.

- NaOCl-sedimented ZN smears alone identified 65% of PTB cases.

- When NaOCl-centrifuged and NaOCl-sedimented sputum was inoculated in LJ media, no growth of *M. tuberculosis* was seen.

7.2 Recommendations

1. 5% NaOCl microscopy method should be introduced as a part of DOTS in countries where culture is not routinely performed.
2. 5% NaOCl microscopy method should be adopted in smear preparation for the laboratory diagnosis of PTB.
3. A single NaOCl-digested smear should be considered by NTP in their case-finding activities.

4. In laboratories where centrifuge are not available, NaOCl-sedimentation method should be an alternative method to NaOCl-centrifugation method.

CHAPTER VIII

8. REFERENCES

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APPENDIX-1

MATERIALS

1. Reagents:

Carbol fuchsin	Loba Chemie Pvt Ltd, Bombay, India
Phenol	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India
Methylene blue	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India
Sodium hydroxide pellets	E Merck Ltd, Mumbai, India
Dehydrated alcohol	Bengal chemicals & pharmaceutical Ltd, Calcutta, India
Sulphuric acid	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India
Sodium hypochlorite	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India

2. Media:

LJ media base	Himedia Laboratories Pvt Ltd, Mumbai, India
Glycerol (98% purified)	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India

3. Materials:

Lysol	Overseas chemical industries, Calcutta, India
Immersion oil	Qualigens Fine Chemicals, Glaxosmithkline Pharmaceutical Limited, Mumbai, India
Plastic centrifuge tubes (20 ml)	Sumitomo Bakelite Co. Ltd
Slides (25cm x 75cm)	Jayna Glass Industries
Gloves	Top Gloves SDN BHD, Malaysia
Slide box	

Bunsen burner

Diamond pen

3. Equipments:

Oven

Binocular microscope

Incubator

Bio-safety cabinet

Bench centrifuge

Inspissator

Refrigerator

Distilled water plant

Autoclave

APPENDIX-2

STAINING REAGENTS AND MEDIA PREPARATION

A. Staining reagents

1. Ziehl-Neelsen stain

i) Ziehl-Neelsen carbol fuchsin (0.3% w/v)

Ingredients	**composition**
Basic fuchsin	*3gm*
Ethyl alcohol (95%)	100ml
Phenol (5%)	45ml/lt
Distilled water	up to 1000ml

ii) Sulphuric acid (20% v/v)

Ingredients	**composition**
Concentrated sulphuric acid	200ml/lt
Distilled water	800ml

iii) Methylene blue (0.1% w/v)

Ingredients	**composition**
Methylene blue	1gm/lt
Distilled water	*1000ml*

B. 4% Sodium hydroxide (NaOH) solution

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

Sodium hydroxide pellet was dissolved in distilled water, then distributed in conical flasks and sterilized by autoclaving at 121°C for 15 minutes.

C. Sterile distilled water

500 ml distilled water was sterile by autoclaving in flasks at 121°C for 15 minutes.

D. Culture media (Lowenstein Jensen media)

a) LJ media base composition

Ingredients	**composition**
L-Asparagine	3.6gm
Monopotassium phosphate	2.4gm
Magnesium sulphate	0.24gm
Magnesium citrate	0.64gm
Potato starch	30.0gm
Malachite green	0.4gm

b) Preparation of Lowenstein-Jensen medium

Ingredients	**composition**
LJ media base	37.5gm
Glycerol	12 ml
Distilled water	600 ml

Procedure:

- 37.5gm of LJ base media was suspended in 600ml of distilled water and was mixed to dissolve it completely.
- The base solution was then sterilized by autoclaving at 121°C for 15 minutes.
- 12 ml of glycerol was added to base solution.
- Fresh hen's eggs were soaked in soap water for 30 minutes and wiped out by cotton.
- Eggs were then scrubbed with spirit and allowed to dry.
- Eggs were broken into plate to check decomposition and then white and yolk were homogenized in sterile blender.
- Homogenized egg was drained through sterile gauge.
- 1000 ml of homogenized whole eggs was added to the autoclaved base solution and mixed thoroughly.

- The complete medium was dispensed in 5 ml volume in sterile tube aseptically.
- It was inspissated at 85°C for 45 minutes in a slanted position.

APPENDIX-3

DEFINATIONS OF TERMINOLOGY USED IN THE STUDY

I Case-detection rate: The number of all new smear-positive cases detected in a certain period out of all new smear-positive cases estimated to occur during that period.

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 two or more initial sputum smear examination positive for AFB or at least one sputum specimen positive for AFB plus sputum culture positive for *M. tuberculosis.*

VI Negative case: A patient with no sputum smear examination positive for AFB and culture negative.

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

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

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

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