CHAPTER 1

1. INTRODUCTION

Tuberculosis is a disease of global importance. One-third of the world population is estimated to have been infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year. The tuberculosis crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. The majority of tuberculosis patients are 15 to 45 years of age, persons in their most productive years of life. Tuberculosis kills over two million people world-wide each year, more than any other single infectious disease, including AIDS and malaria.

Transmission of tuberculosis is virtually entirely by droplet nuclei created through coughing by untreated persons suffering from pulmonary tuberculosis (the most common form) in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons.

Pulmonary tuberculosis usually occurs in the apex of the lungs. These develop cavities which contain large populations of tubercle bacilli that can be detected in a sputum specimen. Pulmonary tuberculosis is suggested by persistent productive cough for three weeks or longer, weight loss, night sweats and chest pain. “The diagnosis can only be made reliably on demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory”(WHO, 1998).

The cornerstone of the laboratory diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Between 5,000 and 10,000 tubercle bacilli per millilitre of sputum are required for direct microscopy to be positive and only a proportion of tuberculosis patients harbour large enough numbers of organisms to be detected in this way. It is also virtually impossible to distinguish different mycobacterial species by microscopy. Patients who have

positive smears carry the greatest number of tubercle bacilli, are the most infectious and are therefore the most important patients to detect early because they are responsible for spreading tuberculosis disease.

Sputum examination by microscopy is relatively quick, easy and inexpensive and must be performed on cases suspected of having tuberculosis. Smear microscopy is also used to monitor treatment progress and control programme outcome.

Examination by bacteriological culture provides the definitive diagnosis of tuberculosis. Depending on the decontamination method and the type of culture medium used, as few as ten viable tubercle bacilli can be detected. However, the usual microbiological techniques of plating clinical material on selective or differential culture media and sub-culturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. Compared to other bacteria which typically reproduce within minutes, *M. tuberculosis* proliferate extremely slowly (generation time 18-24 hours). Furthermore, growth requirements are such that it will not grow on primary isolation on simple chemically defined media. The only media which allow abundant growth of *M. tuberculosis* are egg-enriched media containing glycerol and asparagine, and agar or liquid medium supplemented with serum or bovine albumin.

Culture increases the number of tuberculosis cases found, often by 30-50%, and detects cases earlier, often before they become infectious. Since culture techniques can detect few bacilli, the efficiency of diagnosing failures at the end of treatment can be improved considerably. Culture also provides the necessary material for drug susceptibility testing. Culture of specimens is, however, much more costly than microscopy and requires facilities for media preparation as well as skilled staff (WHO, 1998).

Identification of mycobacteria can be rather complex and needs a multitude of biochemical tests ascertain to which species a mycobacterium belongs. In the context of surveillance of drug resistance, the process can be simplified to a considerable degree as it will only be necessary to decide whether the *Mycobacterium* is a pathogenic species

of the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis,* or *M. africanum*) or not.

Usually, in the context of surveillance of drug resistance, it is only necessary to separate with reasonable certainty *Mycobacterium tuberculosis* complex from environmental mycobacteria, and there is no need to be certain about exact speciation within the complex. In this case a very simple identification procedure suffices.

If it is desirable to identify *M. tuberculosis* complex prior to processing for drug susceptibility testing, the culture, if affluently growing, can be subjected to biochemical tests, i.e., niacin, nitrate reduction, and 68°C labile catalase tests that permit identification of *M. tuberculosis* and *M. bovis.* It should be noted, however, that some strains of *M. tuberculosis* and *M. bovis* are niacin negative. Cultures with too scanty growth for biochemical tests are tested against the anti-tuberculosis drugs isoniazid, rifampicin, streptomycin, and ethambutol plus para-nitrobenzoic acid and thiophene-2 carboxylic acid hydrazide. Almost all strains of *M. tuberculosis* complex are susceptible to para-nitrobenzoic acid and *M. bovis* is susceptible to both para-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. However, identification of *M. bovis* must be ensured by niacin and nitrate reduction tests. In areas of the world where *M. africanum* is prevalent, its proper identification is difficult and not recommended if pyrazinamide susceptibility is not being tested.

If it is considered to be more economical to perform identification and drug susceptibility testing simultaneously, the cultures grown on para-nitrobenzoic acid have to be submitted to biochemical tests (IUALTD, 1998).

CHAPTER 2

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

Isolation and identification of the etiological agent of pulmonary tuberculosis in patients visiting National Tuberculosis Center, Thimi, Bhaktapur.

2.2 SPECIFIC OBJECTIVE

- To perform ZN staining of the sputum samples.
- To perform niacin test, nitrate reduction test, 68ºC labile catalase test and growth on p-nitrobenzoic acid medium.
- To differentiate *M. tuberculosis* from MOTT.

CHAPTER 3

3 LITERATURE REVIEW

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

3.1 EPIDEMIOLOGY

Tuberculosis is a global problem. Although effective methods for its control are available and have been applied successfully for several decades, in some regions the prevalence of tuberculosis is still inordinately high, and a plateau in reduction of incidence is evident. Tuberculosis also remains the leading cause of death among the notifiable infectious diseases. It has also been estimated that someone in the world is infected with TB every second, nearly 1% of the world population is infected with TB every year and according to WHO, one-third of the worlds population is infected with organisms of the *M. tuberculosis* complex, with about 10 million cases of active TB disease reported every year, leading to 3 million deaths annually (Mohan and Sharma, 2001a; Dollin et al, 1994). 95% of TB cases and 98% of TB deaths are in developing countries and these deaths comprise 26% of all avoidable deaths. 75% of TB cases in developing countries are in the economically productive age group (15-50) (Sharma, 2001).

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

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

3.2 DISEASE

Tuberculosis is a chronic bacterial infection caused by *Mycobacterium tuberculosis* (and occasionally by *M. bovis* and *M. africanum*), and characterized by the formation of granulomas in infected tissues and by cell mediated hypersensitivity. Many people refer to the organism, which causes tuberculosis as tubercle bacilli (because they cause a lesion called tubercles) or as acid fast bacilli (AFB) (WHO, 1997). The usual site of disease is the lungs but other organs may be involved. In the absence of effective treatment for the active disease, a chronic wasting course is usual and deaths ultimately supervenes (Daniel, 1994). Incidence of TB depends on the immunity strength of the infected person. It is often the first disease to occur in the AIDS patients, even before any of opportunistic disease appears, and it is generally more intractable than in non-AIDS patients (Alcomo, 1995). It is the leading infections among people with HIV/AIDS. The HIV virus damages the body's immune system and accelerates the speed at which tuberculosis progress from a harmless infection to life threatening tuberculosis. Even in HIV positive cases tuberculosis can be cured if diagnosed in time and treated properly (WHO, 1996). *M. tuberculosis* has been found resistant to several anti-tuberculosis drugs, including two essential first line bacterial drugs: isoniazide and rifampicin, resulting multi-drug resistant tuberculosis (MDR-TB). To make matter worse, global rates of MDR-TB are also in the rise (Duke et al, 2002).

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

The response of a person after exposure to virulent tubercle bacilli depends on interplay of two major immunologic responses: acquired cellular immunity and delayed hypersensitivity. The emergence of hypersensitivity to the proteins of the tubercle bacilli is responsible for the tissue destruction, characteristic of the disease. Sensitization appears about 3 to 4 weeks after infection and is detected by the tuberculin test. Once the person converts to tuberculin-positive reaction he or she usually remains sensitive as long as viable organisms remain in the body.

Individual responses after exposure are thus determined by the previous immunologic experience with the tubercle bacilli. The initial infection with *M. tuberculosis* is referred to as primary infection. Subsequent disease in a previously sensitized person of a primary infection is known as secondary or reinfection tuberculosis.

3.2.1 PRIMARY INFECTION

After inhalation of virulent tubercle bacilli in droplet nuclei, the organisms reach the alveolar spaces where they are phagocytosed by alveolar macrophages. Within the macrophages, bacterial multiplication proceeds with minimal reaction and spreads to the regional nodes in the hilum of the lung and thence to the bloodstream, with a seeding of bacteria in almost all parts of the body. Asymptomatic lymphohematogenous dissemination of the primary infection occurs before the acquisition of tuberculin hypersensitivity and sets the stage for later ‘reactivation’ to present as pulmonary or extrapulmonary disease. Circulating bacilli are efficiently cleared from the bloodstream reticuloendothelial organs, but bacterial multiplication continues in the apices of the lungs and to lesser extent in the kidneys, vascular skeletal areas, and lymph nodes. The

high oxygen tension in the lung apices provide a favorable environment for the organisms and probably account for their predilection for these areas.

About 3 to 4 weeks after infection the development of cellular immunity and tuberculin hypersensitivity greatly alters the course of infection. Activated macrophages limit further bacterial growth and reduce the number of organisms in both primary and metastatic foci.

In approximately 50% of the patients recently infected with tuberculosis some nonspecific clinical symptoms develop at the time of tuberculin conversion. Although undetectable clinically, the hallmark of initial tuberculosis infection is the prominence of hilar adenopathy compared with the relatively insignificant size of initial focus in the lung.

In majority of the patients, the primary lesion heals completely, leading no clinical evidence of prior infection other than hypersensitivity to tuberculin. In some patients, however, the primary infection progresses directly, evolving into a pneumonic caseous process as the organism spread through the bronchi or when a tuberculosis node ruptures into a bronchus. Contiguous spread can cause infection in the pleural and pericardial spaces. In fact, pleurisy is usually abrupt, resembling bacterial pneumonia, with fever, chest pain, and shortness of breath. (Jolik et al, 1992).

3.2.2 TUBERCLE FORMATION

The appearance of hypersensitivity to tuberculin provokes a dramatic change in the host's response to the organisms. The nonspecific inflammatory response evoked on first exposure to tubercle bacilli becomes granulomatous, evoking the formation of tubercles. The tubercle comprises an organized aggregation of enlarged macrophages that, because they resemble epithelial cells, are referred to as epithelioid cells. A peripheral collar of fibroblasts, macrophages, and lymphocytes surrounds the granuloma. Frequently the central region of epithelioid cells undergoes a characteristic

caseous necrosis to produce a ‘soft’ tubercle, the most characteristic hallmark of tuberculosis. When the antigen load at the initial infection site and the regional lymph nodes is large, caseation necrosis may develop and lesions may later calcify. These calcified lesions of the primary site are referred to as the Ghon complex.

After the development of hypersensitivity, the infection becomes quiescent and asymptomatic in the majority of patients (about 90%). In some, however, especially the very young and adults who are immunocompromised or who have other predisposing illnesses, the primary infection may evolve into clinical disease. The progression may be at the site of the primary lesion, or it may be at one or more distant sites where bacilli have arrived during the early hematogenous spread. (Jolik et al,1992).

3.2.3 SECONDARY INFECTION

In a small number of persons whose initial tuberculous infection subsides, secondary disease occurs in spite of acquired cellular immunity. Although the question of whether reinfection tuberculosis results from the breakdown of quiescent foci (endogenous) or from acquisition of new infection from an active case has long been a controversial issue, current opinion favors the endogenous source. In this phase of disease, lesions are usually localized in the apices of the lungs. In about 5% of the patients, apical pulmonary tuberculosis manifests itself within two years of the primary infection. In others, however, clinical disease may evolve many decades later whenever resistance is lowered. Quiescent foci that harbor viable organisms thus remain a potential hazard throughout a person's lifetime.

Because of the acquired cellular immunity, bacilli are more promptly phagocytosized and destroyed by the activated macrophages. As a result, in secondary tuberculosis, lesions remain localized and dissemination of organisms via the lymphatic vessels is usually prevented. Hypersensitivity promotes a more rapid caseation and fibrotic walling-off of the focus. Histologically, the reaction is characteristic of tubercle formation, manifested by a local accumulation of lymphocytes and macrophages. T

lymphocytes and their chemotactic lymphokines play a major role in the development of tuberculous granulomas. The chronocity of these lesions appear to be due to the persistence within them of wax D components of tubercle bacilli (Jolik et al, 1992).

3.3 MORPHOLOGY

M. tuberculosis is a slender, straight, or slightly curved rod with rounded ends. The organism varies in width from 0.3 to 0.6 µm in lengths from 1 to 4 µm. True branching, occasionally seen in old cultures and in smears from caseous lymph nodes, may also be produced in vitro under specific cultural conditions. They are aerobic, non-capsulated, non-motile, and acid and alcohol fast organisms (Ananthanarayan and Paniker, 1996; Forbes et al, 1998). The genus Mycobacterium is the only genus in the family Mycobacteriaceae and it is related to other mycolic acid containing genera (Wayne and Kubica, 1896). The high G+C content of the DNA of Mycobacterium species (61 to 70 mol %, except for *M. leprae* 55 mol%) (Good and Shinnick, 1998) is within the range of those of other mycolic acid containing genera, *Gordonia* (63 to 69 mol %), *Tsukamurella* (68 to 74 mol %), *Nocardia* (64 to 72 mol %) and *Rhodococcus* (63 to 73 mol %) (Goodfellow, 1998).

The bacilli are stained with Ziehl-Neelsen acid fast stain either from cultures or clinical material. With this stain, the bacilli appear as brilliantly staining red rods against a blue background. Organisms in tissue and sputum smears often stain irregularly and have a beaded appearance, presumably because of their vacuoles and polyphosphate content. Although acid fastness of mycobacteria is attributable to their lipid content, the physical integrity of the cell is also essential. The best explanation of the acid fastness of the Mycobacteria is based on a lipid-barrier principle, according to which an increased hydrophobicity of the surface layer follows the complexing of the dye with the mycolic acid residues that are present in the cell wall. This prevents exit of carbol fuchsin that has become trapped in the interior of the cell.

Tubercle bacilli are difficult to stain with Gram stain although they are usually considered to be gram-positive. Staining is poor and irregular because of failure of the dye to penetrate the cell wall. Gram stains of clinical material are thus invalid for the identification of Mycobacteria (Jolik et al, 1992).

The name Mycobacterium meaning fungus like bacterium is derived from mould like appearance of *M. tuberculosis* when growing in liquid media. The genus contains over 40 well defined species including human and animal pathogens and normally saprophytes species. The pathogens include the organism responsible for human and bovine tuberculosis and leprosy. Tubercle bacilli are aerobes, grow slowly with generation time of 12-24 hours, colonies usually appear in 2-3 weeks and may sometimes require 8 weeks incubation, optimum temperature 37ºC and pH 6.4-7.0 (Chakraborty, 2003). The tubercle bacilli able to grow on a wide range of enriched culture media, but Lowstein-Jensen (LJ) medium are widely used. The colonies of *M. tuberculosis* on LJ medium are dry, rough, and creamy or buff coloured (Cheesbrough, 1989).

3.4 TRANSSMISIBILITY

It is well established that patients with sputum that is positive on direct microscopic examination, and thus contains at least 5000 bacilli in 1 ml, are the primary source of infection (Rouillion, Perdrizet and Parrot, 1976). Smear-negative patients, whether culture-positive or not, are of very low infectivity. There has been considerable debate as to whether this relation between smear positivity and infectivity holds true in case of HIV-related tuberculosis as such patients are frequently smear-negative. A study in Kenya however, indicated that pulmonary tuberculosis in HIV-positive person is, in general, as infectious as HIV-negative persons (Nunn et al, 1994).

The risk of infection depends greatly on the closeness of contact as well as infectiousness of the source case. Transmission of tuberculosis occurs principally within households (Van Geuns, Meijer and Styblo, 1975) and other group of people living at

close proximity, such as prisoners and residents of common lodging houses. Children under the age of 3 years are particularly susceptible to infection from source cases, probably owing to closer contact – those exposed to smear positive and negative source cases have, respectively, a 50% and 6% chance of being infected.

There have been reports of ‘explosive’ epidemics of tuberculosis in communities after exposure to highly infectious source cases. In one such case 187 of 3764 children aged between 8 and 11 years became infected after use of a swimming pool supervised by an attendant with open tuberculosis (Rao et al, 1980). Explosive epidemics have occurred in situations, such as hospitals, over crowed accommodations and prisons, where several HIV-positive persons had been exposed to infectious source case (Daley et al, 1992, Edlin et al, 1992, Bouvet et al, 1993).

The average number of persons infected by one source case is expressed as the ‘transmission or contagious parameter’. The number of persons infected by a source case depends on the length of time that the source patient is infectious. In the absence of therapy, about 65% patients with open pulmonary tuberculosis die within 4 years of becoming infectious, with an average survival of 14 months (Springett, 1971). About a quarter recover spontaneously and a minority becomes chronic excretors of tubercle bacilli.

In developing countries, where households are often large and health services suboptimal, the contagion parameter is high: each patient with open tuberculosis infects, on average 20 persons annually. By contrast, patients living under good socioeconomic conditions probably infect only 2 or 3 persons.

Mycobacteria are susceptible to ultraviolet irradiation and outdoor transmission of infection rarely occurs in daylight. Direct sunlight kills tubercle bacilli in 5 minutes (WHO, 2004). Adequate ventilation is the most important measure to reduce the infectiousness of the environment. Most patients become non infectious within 2 weeks

after institution of appropriate chemotherapy because of decrease in the number of organism excreted and a decrease in cough (Daniel, 1994). Tuberculosis is not transmitted by fomites such as dishes and other article used by patients. Sterilization of these articles is of little or no value. Patients with extra pulmonary tuberculosis or smear-negative tuberculosis constitute a minimal hazard for transmission (Park, 2002).

3.5 INCUBATION PERIOD

The time from infection to demonstrable primary lesion or significant tuberculin reaction ranges from 2-10 weeks. While the subsequent risk of pulmonary or extra pulmonary TB is greatest within 5 or two year after infection, latent infection may persist for a lifetime. HIV infection appears to increase the risk greatly and shorten the interval for the development of TB disease (EDCD, 2003).

3.6 DETERMINANTS OF PATHOGENECITY AND PATHOGENESIS

M. tuberculosis produces neither exotoxins nor endotoxins. No single structure, antigen, or mechanism can explain the virulence of the organism. There is likewise no simple in vitro test based on colony morphology or serologic differences that can distinguish virulent tubercle bacilli from its avirulent variant, a distinction that can only be provided by virulence testing in animals. A number of properties, however, are usually associated with the capacity of virulent strains of *M. tuberculosis* to produce progressive disease; although none of these, either singly or together, can account completely for virulence, each undoubtedly plays a crucial role in the pathogenesis of infection.

A number of properties are usually associated with the capacity of virulent strains of *M. tuberculosis* to produce progressive disease. Cord factor and sulphatides are the recognized virulent factors of *M. tuberculosis* (Jolik et al, 1992). Cord factor, a surface glycolipid that causes *M. tuberculosis* in serpentine cords in vitro. Virulent strains of *M. tuberculosis* have cord factor on their surface, where as avirulent strains do not. Second, lipoarabinomannan (LAM) a major heteropolysaccharide similar in structure to the endotoxin of gram negative bacteria, inhibits macrophage activation by interferon-γ

LAM also induces macrophages to secrete TNF-alpha which causes fever, weight loss and tissue damage, and IL-10, which suppresses mycobacteria, induced T-cell proliferation. Third, component activated on the surface of mycobacteria may opsonize the organism and facilitate its uptake by the macrophage complement receptor CR3 (Mac-1integrin) without triggering the respiratory burst necessary to kill the organism. Fourth, a highly immunogenic 65-KD *M. tuberculosis* heat shock protein is similar to human heat shock proteins and may have a role in autoimmune reactions induced by *M. tuberculosis* (Catran et al, 2000). *M. tuberculosis* resides on phagosomes, which are not acidified into lysomes (Clements, 1996). Inhibition of acidification has been associated with urease secreted by mycobacteria and with uptake of mycobacteria by complement or mannose-binding receptors rather than Fc receptor.

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

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

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

The development of the cell mediated or type IV hypersensitivity to the tubercle bacilli probably explains the organism's destructiveness in tissue and also the emergence of

resistance to the organism. The inflammatory response is non-specific resembling the reaction to any form of bacterial invasion. Within 2-3 weeks coincident with the appearance of a positive skin reaction becomes granulomatous and the center of granulomatous becomes caseous, forming “soft tubercle”. The pattern of host response depends on whether the infection represents a primary first exposure to the organism or secondary reaction in an already sensitized host (Catran et al, 2000).

3.7 GENETICS

Genetic studies on mycobacteria have been greatly hampered by the slow growth of the organism. Although the existence of genetic variation within the members of the group is well recognized, only in the case of drug resistance have the observed phenomenon been subjected to critical genetic analysis. With the use of fluctuation test analysis for the calculation of mutation rates, mutations are observed to occur in *M. tuberculosis* at very low frequencies comparable to those of other bacteria.

Another commonly observed class of spontaneous mutation is that causing alterations in colony morphology. Different colony types are observable between strains as well as within the species. Smooth (S) and rough (R) variants of tubercle bacilli have been described. With few exceptions, virulent organisms produce R colonies, but avirulent strains may also produce colonies of R type. The best example of this is the H37 strain of *M. tuberculosis* which, by manipulations of culture conditions, has been dissociated into the rough virulent (Rv) and rough avirulent (Ra) variants. Although both variants are rough, their colonial morohology on both egg and liquid media is different and characteristic. These classic strains have been extensively used in experimental studies for more than 50 years.

Mycobacterial chromosomes were found to have molecular weight of (2.5-5.55) $X10^8$, with those of the major pathogen *M. tuberculosis* being at the lower end of range. In addition to main chromosome, some strains contain one or more plasmid (Grange, 1990).

3.8 DIAGNOSIS

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

The timely identification of a person infected with *M. tuberculosis* and their rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measure for the control of the TB (Noorden and Godal, 1988). Diagnostic testing for both latent tuberculosis infection (LTBI) and active disease has changed little during last century. LTBI, until very recently, has been diagnosed exclusively by the tuberculin skin test (Brodie and Shulunger, 2005). Diagnosis of active disease includes clinical suspicion, chest radiographs, staining for acid-fast bacilli, culture for mycobacteria, and more recently, nucleic acid amplification assay (Foulds and O`Brien, 1998).

Ideal TB diagnosis should address the following four focal aspects of TB control in low-income countries.

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

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

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

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

3.8.1 LABORATORY DIAGNOSIS

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

1. Detection and isolation of mycobacteria

2. Identification of species of the isolate

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

Microscopical Examination

The detection of acid-fast bacilli in stained smears is the easiest and most rapid procedure for evaluating a clinical specimen. Since most patients with symptomatic tuberculosis will demonstrate acid-fast bacilli in the sputum, sputum examination play an important role in tuberculosis control program.

For making the smear, small caseous areas of the sputum should be selected, spread in a thin layer on a new slide, and stained with Zeil-Neelsen or Kinyoun stain. Where facilities are available, fluorescent staining with auramine O or rhodomine facilitates more rapid scanning of a sputum smear. A recommended method for examining the smear is to make three longitudinal sweeps of the stained area, parallel to the length of the slide. A report from the laboratory should provide an estimate of number of acid fast bacilli detected.

The cornerstone of the laboratory diagnosis of TB is direct microscopic examination of appropriately stained specimens for tubercle bacilli. Between 5000 and 10000 tubercle bacilli per milliliter of sputum are required for direct microscopy to be positive and only a proportion of tuberculosis patients harbor large enough numbers of organisms to be detected in this way. It is also virtually impossible to distinguish different mycobacterial species by microscopy. Patients who have positive smears carry the greatest number of tubercle bacilli, are the most infectious and are therefore the most important patients to detect early because they are responsible for spreading tuberculosis disease.

Sputum examination by microscopy is relatively quick, easy and inexpensive and must be performed on cases suspected of having TB. Smear microscopy is also used to monitor treatment progress and control program outcome.

Examination by bacteriological culture provides the definitive diagnosis of TB. Depending upon the decontamination method and the type of culture media used, as few as ten viable tubercle bacilli can be detected. However, the usual microbiological techniques of plating clinical material on selective or differential cultural media and sub-culturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. Compared to other bacteria which typically reproduce within minutes, *M. tuberculosis* proliferate extremely slowly (generation time 18-24 hours). Furthermore, growth requirements are such that it will not grow on primary isolation on simple chemically defined media. The only media which allow abundant growth of *M. tuberculosis* are egg-enriched media containing glycerol and asparagines, and agar or liquid medium supplemented with serum or bovine albumin.

Culture

Cultural techniques are more sensitive than the microscopy and may detect as few as 10-100 organisms per ml of specimen. Lowenstein-Jensen medium, a solid egg-based medium containing glycerol is widely used for the isolation of *M. tuberculosis* and similar media containing pyruvic acid in place of glycerol are used for the isolation of

M. bovis. Liquid media are used in the radiometric methods. Biphasic systems containing broth and slides coated with solid agar-based media are commercially available. These permit bacterial growth to be detected more rapidly than by conventional methods but not as rapidly as radiometric techniques (Salfinger, Demchik and Kafader, 1990).

Inoculated culture media are usually incubated for at least 8 weeks and inspected weekly for growth. Most strains of the *M. tuberculosis* complex produce visible colonies within 4 weeks, but growth may be delayed if the patient has received antituberculosis drugs. Cultures should be incubated at 35ºC. Colonies appearing on the medium are shown to be mycobacteria by means of Ziehl-Neelsen staining and are usually identified by simple cultural and biochemical tests. Thus, members of the *M. tuberculosis* complex are clearly identifiable by their slow growth rate, lack of pigment, failure to grow at 25ºC and sensitivity to p-nitrobenzoic acid (Collins, Grange and Yates, 1985). Nucleic acid probes for the rapid identification of this complex and, specially, *M. tuberculosis* are commercially available.

Culture increases the number of tuberculosis cases found, often by 30-50%, and detects cases earlier, often before they become infectious. Since culture techniques can detect few bacilli, the efficiency of diagnosing failures at the end of treatment can be improved considerably. Culture also provides the necessary material for the drug sensitivity testing. Culture of specimens is, however, much more costly than microscopy and requires facilities for media preparation as well as skilled staff.

Culture should be used selectively, in the following order of priority:

1. Surveillance of tuberculosis drug resistance as an integral part of evaluation of control program performance.

2. Diagnosis of cases with clinical and radiological signs of pulmonary tuberculosis where smears are repeatedly negative.

3. Diagnosis of extra-pulmonary and childhood tuberculosis.

4. Follow-up of tuberculosis cases who fail a standardized course of treatment and why may be at risk of harboring drug resistant organisms.

5. Investigation of high-risk individuals who are symptomatic, eg. Laboratory workers, health care workers looking after multi-drug resistant patients.

There are three types of culture media: solid media, which include egg-based media (LJ, Ogawa medium) and agar based media (Middlebrook 7H10 and 7H11), and liquid media (Middlebrook 7H12 and other liquid broths). Solid media, long the standard for culturing the mycobacteria, are slower than liquid media, which are now widely used alongside solid media to increase sensitivity and decrease recovery time (Morgan et al, 1983; Sharp et al, 2000). In fact, LJ, 7H10, and 7H11 media may detect mycobacteria in less than 4 weeks, but they require incubation as long as 6 to 8 weeks before they are classified as negative (Morgan et al, 1983). The LJ medium contains eggs, glycerol, asparagines, mineral salts and malachite green dye (that inhibits certain contaminating bacteria). LJ medium is popular for isolating human strain of *M. tuberculosis* and most other mycobacteria (Salfinger et al, 1990). Ogawa medium that is also in common use contains egg yolk instead of whole egg (Grange, 1990). The presence of glycerol in LJ medium improves the growth of *M. tuberculosis*, but not that of *M. bovis* . Addition of sodium pyruvate, on the other hand, increases the growth of *M. bovis* and some strain of drug resistance *M. tuberculosis* (Watt et al, 1996).

M. tuberculosis is an obligate aerobe, grows optimally at 37ºC and pH 6.4-7.0. On solid medium it forms a dry, rough, raised, irregular, colony with wrinkled surface. The colonies are creamy white initially, becoming yellowish or buff colored later and tough

when picked off. They are tenacious and not easily emulsified. Until, recently, identification of *M. tuberculosis* from positive cultures depend on biochemical tests for niacin, aryl sulphatase, neutral red, catalase-peroxidase, amidase, and nitrate reductase after incubation for 2 to 3 weeks (Forbes et al, 2000).

In contrast, broth media combined with DNA probes for rapid species identification typically provide result in less than 2 week with smear positive samples and somewhat longer with smear negative samples (Morgan et al, 1983; Kanchana et al, 2000). Broth media formulations include both manual and automated systems using radiometric and colorometric methods for detection of mycobacteria. Examples of broth media include the BACTEC 460TB and BACTEC MB9000 radiometric methods, the Mycobacterial Growth Indicator Tube or MGIT non-radiometric method, and the manual Septic-Check AFB System (Brodie and Schlunger, 2005).

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

Cultures of mycobacteria can also be identified with reverse-phase high performance liquid chromatography. Chromatography techniques are quite accurate and are also able to identify many mycobacterial species. The chromatography method is based on the analysis of fatty acid extracted from the mycobacterial cell wall and methylated to form volatile esters (Smid and Salfinger, 1994).

3.8.2. DIFFERENTIATION OF *M. TUBERCULOSIS*.

For the preliminary identification of tubercle bacilli the following characteristics apply:

(a) Tubercle bacilli do not grow in primary culture in less than 3 to 4 weeks to give visible growth.
(b) The colonies are buffed colored (never yellow) and rough, having appearance of bread crumbs or cauliflower.
(c) They do not emulsify in saline used for making smear but give a granular suspension.
(d) Microscopically, they are frequently arranged in serpentine cords of varying length or show district linear clumping. Individual cells are between 3 and 4 micrometer in length.

Although a presumptive diagnosis of tuberculosis may be made by an experienced laboratory technologist on the basis of the characteristics of tubercle bacilli, it is best to do confirmatory tests. Unfortunately, there is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Nevertheless the following tests, when used in combination with the characteristics described before will enable the precise identification of >95% *M. tuberculosis*.

3.8.2.1 NIACIN TEST

Pope and Smith in 1946 reported the great differences between bovine and human tubercle bacilli with regard to the production of B-complex vitamins. Kenne in1956 developed niacin test for identifying human strain of *M. tuberculosis* and since then many modifications and improvements have been suggested. This method is based on the detection of nicotinic acid a metabolite, which appears in cultures due to the lack of an enzyme that converts niacin to another metabolite in the coenzyme pathway. Human strain is found to synthesize seven to eight times greater quantity than other mycobacteria (Boisvert, 1960). The cultures are grown for four weeks and autoclaved to extract the nicotinic acid. Two drops of the extract are mixed with three drops of 10% 1w/w aqueous solution of cyanogens bromide and a similar quantity of 3% Benzidine in alcohol (Medveczky, 1960) in a depression of porcelain spot test plate. A positive test is

indicated by bright pink colour. Niacin test is considered a fairly reliable test to differentiate human strain from bovine, avain and other anonymous mycobacteria.

Niacin (nicotinic acid) plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all mycobacteria. Although all mycobacteria produce niacin, comparative studies have shown that because of a blocked metabolic pathways *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests.

Cultures grown on egg medium yield the most consistent results in the niacin test and LJ medium is therefore recommended. A culture must at least 3-4 weeks old and must have sufficient growth of more than 50 colonies. Because *M. tuberculosis* excretes niacin in the growth medium, cultures with confluent growth may give a false-negative niacin reaction because the extracting fluid cannot come in contact with the culture medium. When this occurs, expose the underlying medium surface away or puncturing through some of the culture growth.

Aeration of cultures intended for niacin testing is very important. Caps should be loose on slants throughout the entire incubation period and special Cap-o-Test stoppers are recommended.

INH test strips are also used for detecting isonicotinic acid (Niacin) and its metabolite in the aqueous extract of organism and medium. The INH test strips are absorbent paper strips impregnated with chloramines T, potassium thiocyanate, citric acid and barbituric acid. The pyridine ring of isonicotinic acid splits the cyanogen bromide to form a glutaconaldehyde derivative. The derivative condenses with barbituric acid to form blue-purple polymethine dye.

Paper test strips for the detection of niacin are commercially available. They compare well to the chemical reagents in detecting niacin production. A paper strip method obviates the need to prepare and store the unstable and toxic chemical used to demonstrate the presence of niacin, but is much more expensive (WHO, 1998).

Since about 1960 the niacin test has been the most widely used laboratory procedure for differential identification of the mycobacteria. Pope and Smith, Bird, and Konno et al, have demonstrated that human tubercle bacilli produce considerably more niacin than other mycobacteria. This characteristic is sufficiently regular for human strains to be accurately differentiated from other acid fact organisms. Several modifications of the niacin test have been made to increase sensitivity, ease performance, or both all are based on the reaction of niacin with a cyanogenhalide, usually in the presence of a primary amine. In most tests, niacin is extracted from a mature culture on a solid medium with a small volume of water or saline. In the procedure described by Runyon, equal volumes of an aqueous extract of a culture, 4% ethanolic aniline, and 10% cyanogen bromide are mixed in a test tube in a fume hood. Alternatively, chloramine-T and sodium or potassium cyanide may be used and the unstable ethanolic amine omitted, although this modification results in a somewhat less sensitive reaction. The Runyon procedure is the most widely used in this country but has several significant procedural disadvantages. Ethanolic aniline is particularly sensitive to moisture and light, and cyanogen bromide when vaporized is a potent irritant and is extremely toxic. Its hydrolysis product, hydrocyanicacid, is also highly toxic.

Kilburn and Kubica have described a test for niacin production by mycobacteria in which reagents are dried on a strip of filter paper. This lessens or eliminates many of the hazards and inconveniences previously associated with the niacin test. The present report describes the development of a reagent-impregnated test strip with improved stability, sensitivity, and ease of use (PathoTec-Niacin; General Diagnostics Division,

Warner-Lambert Co., Morris Plains, N.J.). Data are presented which demonstrate a high degree of correlation between the results of conventional and strip tests in the identification of mycobacteria.

Three hundred seventy-eight cultures of mycobacteria were tested for niacin production with four-zone test strips and by the cyanogen bromide-aniline method of Runyon. Fresh isolates were grown for 3 to 6 weeks on Middlebrook7H10 agar or Lowenstein-Jensen egg medium water. Approximately 0.6 ml of extract was placed in a screw-cap tube (13 by 100 mm), a test strip was added in such fashion that the end opposite the orange marker was immersed in the extract, and the tube was closed securely with a cap having a rubber liner. The system was allowed to react at room temperature for15 to 20 min with occasional gentle agitation and was observed bromide (10%) and 5% alcoholic aniline were added to a second sample of extract and similarly observed for color development. After use, the entire closed system was autoclaved and discarded. Cultures which were positive by the strip test but negative by the Runyon method were retested by substitution of 3% benzidine base in 95% ethanol for the aniline reagent or by repetition of the Runyon procedure after sufficient reincubation to give a total incubation time of 4 weeks or more. Niacin-negative cultures were identified as strains other than *M. tuberculosis* by means of additional differential tests (Young et al, 1970).

Niacin extractions from *M. tuberculosis* growing on 7H-10 or 7H-11 agar base medium yield consistent results when performed at 37°C for 2 h. The technique does not require any modification of the media formulation.

It is reported that when an agar base medium such as Middlebrook-Cohn 7H-10 or 7H-11 is used for the isolation and identification of *M. tuberculosis*, 0.1% L-potassium aspartate or 0.25% L-asparagine should beadded to the medium to provide for consistent niacin test results. Unfortunately, the addition of potassium aspartate to the medium negates its use for susceptibility testing, because aspartic acid increases the in vitro minimal inhibitory concentration of isoniazid and streptomycin. Also, asparagine,

in the recommended concentration, is inhibitory to some strains of *M. tuberculosis*. Furthermore, neither of the supplemented media is readily available from commercial sources.

In a limited study, it was found that the problem of inconsistent niacin test results was not one of niacin production, but one of niacin extraction. Thus, the study reported here is an extension of that preliminary report to evaluate the modified extraction procedure. This study includes the use of more strains of *M. tuberculosis* plus the addition of 7H-11 medium, as well as the 7H-10 medium as used in the initial study. The inocula used in this study consisted of a suspension of organisms, which were made by removing portions of several colonies of *M .tuberculosis* growing on Lowenstein-Jensen medium and by emulsifying them in 5 ml of sterile saline.

The inocula of the negative control cultures, *M. kansasii*, *M. avium-intracellulare*, and *M. fortuitum*, growing on Lowenstein-Jensen medium were prepared in a similar manner. The 7H-10 and 7H-11 media were inoculated with 0.2ml of the suspension and incubated in an 8 to10% concentration of CO_2 at 37°C until there was sufficient growth to test for the presence of niacin. Niacin extraction consisted of flooding the slant cultures with approximately 2 ml of sterile saline and holding the cultures in a near horizontal position for 2 h at 37°C.

A total of 713 isolates of *M. tuberculosis* and 28 negative control cultures growing on 7H-10and Lowenstein-Jensen media were tested for the presence of niacin by a method previously described, which utilized benzidine-cyanogen bromide. Because of the carcinogenic nature of benzidine, the aniline-cyanogen bromide method was used to test 115 isolates of *M. tuberculosis* and 28 negative control cultures growing on 7H-11 and Lowenstein-Jensen media.

Of the 713 isolates grown on 7H-10, 706 (99.0%) were niacin positive on initial testing by the modified technique. The remaining seven cultures were niacin positive on retest

after 1 additional week of incubation. The expected negative niacin test results were obtained from the 50 niacin-negative controls on both the 7H-10 and 7H-11 media. There was also an indication that all 115 of the *M. tuberculosis* isolates grown on 7H-11 were niacin positive when tested by the modified technique and that all of the niacin test results reported in this study were confirmed by the standard method when the organisms were grown on Lowenstein-Jensen slants.

The consistent niacin test results obtained from *M. tuberculosis* when grown on 7H-10 and 7H-11, using the modified niacin extraction procedure, indicate the reliability of the procedure. Although the modified procedure requires an extraction time of 2 h at 37ºC, it has certain advantages over those which require a modification of the media: (i) the prepared agar base media (7H-10 and 7H-11) are readily available from commercial sources; (ii) it can be performed in any laboratory and is not limited to those having a media kitchen; (iii) if media are prepared in-house, the same agar base medium can be used in susceptibility tests in both the control and drug-containing quadrants; and (iv) the procedure does not influence the growth pattern of the mycobacteria. (Neimester , 1982).

3.8.2.2 NITRATE REDUCTIOM TEST

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allows this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from other mycobacteria. This test is based on the principle that the enzyme nitrate reductase causes the reduction of the nitrate in the presence of suitable electron donor, to nitrite or nitrogen.

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allows this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from other mycobacteria. This test is based on the principle that the enzyme nitrate reductase causes the reduction of nitrate, in presence of a suitable

electron donor, to nitrite or nitrogen. Nitrate reduction test, the presence of nitrite (Product of the nitroreductase enzyme) is detected. *M. tuberculosis* is suspended in a buffer solution containing nitrate and incubated at 37ºC for two hours reduces nitrate to nitrite which give a pink or red colour when treated with sulphanilamide and N-naphthylethylene diamine dihydrochloride.

This test detects the ability of mycobacteria to reduce nitrate to nitrites. *M. tuberculosis* and H37RV strain are the nitrate reduction positive whereas *M. bovis* and *M. intracellularae* are nitrate reduction negative.

Now-a-days, paper strips for the detection of nitrate reduction are commercially available. The paper strips for test method yields most consistent results with mycobacterium that vigorously reduce nitrate, such as *M. tuberculosis*. It therefore provides reliable results and is much less labour-intensive than the chemical method, but is expensive (WHO, 1998).

Cultures to be tested for nitrate reduction should be 4 weeks old and have abundant growth on Lowenstein Jensen medium are recommended.

The nitrate reductase test is one of a battery of biochemical tests used to identify members of the genus *Mycobacterium*. In conjunction with other tests, nitrate reductase is useful to distinguish *M. szulgai* from *M. scrofulaceum* and *M. gordonae*, *M. kansasii* from *M. marinum.*, *M. fortuituim* from *M. chelon*ei, and *M. tuberculosis* from *M. simiae* and *M. bovis*. However, the test is not without problems. Reagents used in the conventional test have a short shelf life and must be prepared in-house. The test can be difficult to read because the positive reaction color can flash instantly or quickly fade. Commercially available paper strip reagents have a longer shelf life, but they usually are positive only for mycobacteria with strong nitrate reductase activity.

A new crystalline reagent for nitrate reductase tests was compared with standard liquid reagents on 437 strains of mycobacteria. The results for isolates of *M.* avium complex, *M. kansasii, M. gordonae, M. scrofulaceuM., M. fortuituM.*, and *M. chelonei* agreed 100% with the expected results. Of the 177 *M. tuberculosis* isolates, 4 were negative by the conventional method. Two of these four isolates were positive with the new reagent. Of the positive nitrate tests carried out with liquid reagents, 42% flashed instantly or faded in color; none of the tests carried out with the new crystalline reagent flashed or faded. A stronger color reaction was seen for 28% of the positive tests with the new reagent.

Recently, Lampe described a crystalline reagent that has a long and stable shelf life and that was used to detect nitrate reductase. Although reliable results were obtained with 135 bacterial strains, only 8 strains of mycobacteria, representing four species, were tested. In addition, the method used in that study differed from the conventional mycobacterial nitrate reductase test. The purpose of this study was to use conventional mycobacterial nitrate reductase test procedures to compare the crystalline and liquid reagents.

Nitrate reductase test reagents. The convention all liquid nitrate reagents are a 1:2 dilution of concentrated hydrochloric acid, a 0.2% solution of sulfanilamide in distilled water, and a 0.1% solution of N-(one naphthyl) ethylenediamine dihydrochloride in distilled water. The new reagent described by Lampe consists of one part sulfanilic acid, one part N-(one naphthyl) ethylenediamine dihydrochloride, and 10 parts L-(+)-tartaric acid. All chemicals were obtained 546.from Sigma Chemical, Co., St. Louis, Mo. The chemicals were put in a dark bottle and were mixed by vigorous manual shaking about 30 times. The mixture had a heterogeneous crystalline appearance. The shelf life of the reagent was reported to be 6 months; our mixture was stable for 6 months, at which time the supply was exhausted.

Nitrate reductase tests. All mycobacteria were tested for nitrate reductase activity by using a slight modification of the conventional combined niacin-nitrate test. Two milliliters of the conventional sodium nitrate solution was added to an actively growing mycobacterial culture that was then incubated at 36ºC for 2 h. After incubation, a 0.5-ml portion of the liquid was withdrawn and placed in a sterile, screw-capped tube. With a spatula, a small amount of the crystalline reagent was added to the solution (the quantity of reagent was not critical). The test was read as positive when a pink to deep red color developed. The remaining 1.5 ml of substrate solution was used for the conventional niacin and nitrate tests. All positive reactions were compared with color standards. A "flash in color" was defined as instantaneous color loss, and "fading" was defined as a gradual loss of color within 10 min. For either nitrate test, zinc powder was added to the tubes that developed no color to confirm the negative test result. Isolates of *M.* that had a nitrate reductase test result that differed from the expected result were retested.

The results of nitrate reductase tests with the conventional and crystalline reagents are grouped according to the anticipated results and listed the mycobacteria that were positive for nitratereductase. The test results for isolates of *M. kansasii, M. fortuitum, M. szulgai,* and *M. flavescens* and reference cultures of *M. tuberculosis* agreed 100% with the expected results. Four clinical isolates of *M. tuberculosis* were negative by conventional reagent tests; of these isolates, two strains were positive when the crystalline reagent was used. The remaining two Isolates were confirmed as nitrate reductase-negative *M. tuberculosis* by the Centers for Disease Control. No mycobacteria were falsely positive for nitrate reductase when either the liquid or the crystalline reagents were used. Variable reactions were observed with members of the *M. terrae* complex. Of 36 isolates, 26 (72%) gave a positive reaction with the liquid reagent, whereas 29 of 36 isolates (81%) were positive when the crystalline reagent was used.

Of the 226 tests that were positive with the conventional liquid reagent, 34 (15%) flashed and 60 (27%) faded in color, which made the test difficult to interpret 45% of

the time. None of the tests carried out with crystalline reagent flashed or faded. The color reaction was noted to be more intense 28% of the time when the crystalline reagent was used (Warren et al, 1983).

The activities of the nitrate reductase enzyme of *M. tuberculosis*, *M. bovis*, and of BCG were assayed with and without addition of electron donors. *M. tuberculosis* always reduced nitrate; *M. bovis* did so only in the presence of electron donors, and BCG did not show enzymatic activity.

One of the most frequently used methods for differentiating *Mycobacterium bovis* and *M. tuberculosis* is enzymatic reduction of nitrate to nitrite; this test is negative for *M. bovis* and positive for *M. tuberculosis* Bonicke et al, assayed this technique in the presence of various electron donors in two standard strains of *M. tuberculosis* and one of BCG.

The purpose of this study was to establish the value of the above enzymatic test when applied to wild species of *M. bovis* and *M. tuberculosis* in the presence of fatty acids and other related compounds. The behavior of the Pasteur strain of BCG was also studied under identical conditions.

Twenty-seven strains of *M. tuberculosis* from secretions of patients, BCG (Pasteur strain), all cultivated in Lowenstein-Jensen medium, and 20 strains of *M. bovis* from bovine lymph nodes, cultivated in Stonebrink medium, were used. From well-developed cultures, colonies were collected with loops; suspensions were then prepared in 0.0667 M phosphate buffer at pH 7.0 and agitated in flasks with glass balls. Concentration was controlled with a standard suspension containing 1 mg of mycobacteria per ml.

The technique was developed as described by Bonicke et al. The concentration of the nitrite formed was determined colorimetrically by using a spectrophotometer (Coleman Instrument Corp., model 6A) and a standard absorbance concentration curve. Reaction

times were 3 and 24 h. The electron donor compounds used were: acetic acid, propionic acid, pyruvic acid, DL-alarine, DL-serine, and Tween 80. The pH of all solutions was adjusted at 7.0. Lactic acid was not included in the study in view of the limited increase in the enzymatic activity it produces on *M. tuberculosis* GRW, as stated before.

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The BCG strain failed to reduce nitrate under all the conditions of the experiment and time periods.

All the *M. tuberculosis* strains tested showed some nitrate reductase activity after 3 h of incubation, and all showed an increase in this activity in the reading of the reaction 24 h after incubation, in the presence or absence of reducing compounds. None of the *M. bovis* strains assayed reduced nitrate enzymatically without adding such compounds, even after 24 h of incubation. On the other hand, all these strains showed measurable enzymatic activity in the presence of some of the compounds. Of these, pyruvic acid had the most marked effect on the nitrate reductase activity of *M. bovis* and *M. tuberculosis.*

Although the amount of nitrite formed in the 20 wild strains of *M. bovis* varied greatly from one strain to another, at the 24-h reading the percentages of the strains that produced measurable quantities of nitrite in the presence of acetic acid, pyruvic acid, DL-alanine, and Tween 80 were 90, 100, 95, and 70%, respectively. Bonicke et al, have stated in their paper that bovine and BCG strains remain incapable of reducing nitrate, even in the presence of such compounds. Apparently, Argentine wild bovine strains would have a different behavior from that of the collection strains used by the above-mentioned authors.

The results differ quantitatively from those obtained by Bonicke et al. These differences may be accounted for by our use in this study of a bacillary suspension of 1 mg/ml and wild strains, as compared with a suspension of 10 mg/ml and collection strains used in the original study.

The results of our experiments showed that *M. tuberculosis*, *M. bovis*, and the BCG strains behave differently, a fact which can be useful for their identification in the laboratory: *M. tuberculosis* reduces nitrate even in the absence of compounds that can act as electron donors; *M. bovis* does so only in the presence of such compounds, and BCG (Pasteur strain) does not show this enzymatic activity in any conditions (Escoto and Kantor, 1978)

3.8.2.3 CATALASE TEST

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all posses catalase enzyme, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis.*

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

Reduced flavoprotein reacts directly with gaseous oxygen to form hydrogen peroxide which is an oxidative end product of the aerobic breakdown of sugars (Mackie and McCartney, 1999).

FPH_2 (Reduced flavoprotein) + O_2 → FP (Oxidised flavoprotein) + H_2O_2

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

$2\ H_2O_2 \rightarrow 2H_2O + O_2$

This test is usually performed by suspending colonies of growth in a mixture of tween 80 and hydrogen peroxide, the appearance of bubbles is interpreted as a positive reaction. Mycobacteria usually have catalase activity. *M. tuberculosis* has moderate catalase activity, while saprophytes and the unclassified mycobacteria have very high activity. A character which is often helpful in identification of strains of *M. tuberculosis* with high degree of isoniazid-resistant is usually catalse negative, a further differentiating feature. Antigenically, catalase extracted from *M. tuberculosis* similar to that obtained from *M. bovis* but distinct from that of *M. avium.*

Catalase test at 68ºC: The growth is incubated in phosphate buffer pH 7 at 68ºC in a water bath for 20 minutes and cooled to room temperature. Tween peroxide is then added. The appearance of bubbles indicates a positive reaction. *M. tuberculosis* and *M. bovis* gives negative reaction, while positive reactions are usually obtained with several other mycobacteria.

Mycobacteria posses several kinds of catalase that vary in heat stability. Quantitative differences in catalase activity can be demonstrated by one or more of the following tests

Room temperature or drop method (indicates the presence of catalyse)
Semiquantitave test (indicates level of catalyse production)
68ºC test at pH 7 (indicates loss of catalyse activity due to heat)

Drug susceptible strains of *M. tuberculosis* do form catalyse as indicated by the drop method, produce less than 45mm of bubbles in the semiquantitative test and lose catalase activity when heated to 68ºC for 20 minutes. For these tests 14 day old cultures on LJ butts should be used, i.e. the media tubes should be inspissated in an upright position to provide a butt and should not be slanted. The tubes must have stoppers

which permit exchange of air, e.g. Cap-o-Test stoppers. The cultures should be incubated in a well-humified incubator at 35ºC-37ºC, with loose caps, for 14 days (WHO, 1998).

In recent work with 11 species of Acetobacter were observed that very erratic results were obtained when cultures were subjected to the 'catalase test'. When well-developed colonies which had been growing at 250 on malt-extract agar (pH 5-0) in Petri dishes for 14 days were treated with 0.5N H_20_2, three species gave no response and some of the others reacted only weakly. The results obtained with cultures 8 days' old under conditions which otherwise were identical were practically the same (Walker and Tosic, 1942)

M. tuberculosis has a relatively high resistance to killing by hydrogen peroxide and organic peroxides. Resistance may be mediated by mycobacterial catalase-peroxidase (KatG) and possibly by alkylhydroperoxide reductase (AhpC). To determine the interrelationship between sensitivity to H_20_2, catalase and peroxidase activities, and bacillary growth rates measured both intracellularly in human monocytes and in culture medium, we examined one laboratory strain, two clinical isolates, and three recombinant strains of *M. tuberculosis* with differing levels of KatG and AhpC. Five of the mycobacterial strains had intracellular doubling times of 27 to 32 h, while one KatG-deficient clinical isolate (ATCC 35825) doubled in; 76 h. killing of mycobacteria by exogenously added H_20_2 was more pronounced for intracellular bacilli than for those bacilli derived from disrupted monocytes. Strains with no detectable KatG expression or catalase activity were relatively sensitive to killing (43 to 67% killing) by exogenous H_20_2. However, once even minimal catalase activity was present, mycobacterial catalase activity over a 10-fold range (0.56 to 6.2 μ/mg) was associated with survival of 85% of the bacilli. Peroxidase activity levels correlated significantly with resistance of the mycobacterial strains to H_20_2-mediated killing. An endogenous oxidative burst induction by 4b-phorbol 12b-myristate13a-acetate treatment of infected monocytes

reduced the viability of the KatG null strain (H37RvInhr) but not the KatG-over expressing strain [H37Rv (pMH59)]. These results suggest that mycobacterial *resistance* to oxidative metabolites (including H_2O_2 and other peroxides) may be an important mechanism of bacillary survival within the host phagocyte (Manca et al, 1999).

M. tuberculosis is a facultative intracellular bacterium which has evolved sophisticated mechanisms to allow it to survive inside host mononuclear phagocytes. Once phagocytosed, the organism resides in a vacuole which does not fully mature along the endocytic pathway. Within the vacuole, the organism must protect itself against intracellular bactericidal mechanisms, including the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates which diffuse freely through the cell.

M. tuberculosis has been shown to have a high resistance to killing by up to millimolar concentrations of H_2O_2. This resistance is believed to be mediated by the sole mycobacterial catalase-peroxidase protein (KatG) and the alkyl hydroperoxide reductase protein (AhpC), encoded by the genes *katG* and *ahpC*, respectively. Isoniazid, a widely used frontline antimycobacterial agent, requires activation by KatG before exerting a lethal effect.

Isoniazid resistance in a majority of clinical isolates results from point mutations in *katG*. Isoniazid-resistant mutants selected in vitro frequently lose KatG entirely. The availability of KatG mutant organisms has facilitated investigations of the role of catalase peroxidase in the virulence of *M. tuberculosis*. These studies have produced conflicting results. Some investigators have observed no correlation between loss of KatG activity and virulence of *M. tuberculosis* in mice and guinea pigs and no correlation between KatG levels and susceptibility to killing by hydrogen peroxide. However, others have found a strong apparent correlation between KatG status and *M. tuberculosis* virulence. More recently, the loss of catalase and peroxidase activities in

M. bovis has been shown to correlate with the lack of virulence of *M. bovis* in guinea pigs. Reintroduction of a functional *katG* into this strain restored both isoniazid sensitivity and virulence in the host animal (Manca et al, 1998)

3.8.2.4 GROWTH ON MEDIUM CONTAINING P-NITROBENZOIC ACID (PNB)

Human and bovine type of tubercle bacilli can be differentiated from all other mycobacteria in their inability to grow in LJ medium containing 500µg/ml Para-nitro-benzoic acid. Occasionally, human strain may give faint growth in this medium. The result of this and niacin test will help typing human and bovine strains (National TB institute, India, 1998).

In laboratories where facilities and reagents for niacin testing are not available, identification of tubercle bacilli may be done by the combination of one or more of catalase tests described previously together with growth at 25ºC on LJ medium and growth on LJ medium containing p-nitrobenzoic acid at 37ºC.Problems with incubation at 25ºC may be encountered in tropical regions. A refrigerated incubator should be used where available; as an alternative, a water bath within a refrigerator or cold room should be used.

In screening for anonymous mycobacteria the use of thiosemicarbazone (10µg. /ml.) may give rise to false positive results. Although these can usually be overcome by further tests, additional time and effort is required, which seriously limits the value of thiosemicarbazone as a true screening agent. Para-nitrobenzoic acid (500 µg/ml.) has proved more satisfactory in our hands; no false positive results have been obtained and it has the additional advantage that strains of *M. kansasii* (usually thiosemicarbazone sensitive) are resistant to para-nitrobenzoic acid.

In recent years there has been increasing interest in human infections with mycobacteria other than *M. tuberculosis* and various tests have been suggested to enable laboratories to recognize such strains. As almost all anonymous mycobacteria are resistant to one or more of the standard anti-tuberculosis drugs it is of particular importance that they should be separated from drug-resistant strains of *M. tuberculosis*. When, in 1963, the Western Regional Hospital Board established a reference laboratory to maintain a register of patients in the west of Scotland who were excreting drug-resistant mycobacteria, one of its first tasks was to undertake such a classification. The screening procedure suggested by Marks and Trollope (1960) has the advantage of speed and technical simplicity and has been used in conjunction with each sensitivity test performed by this laboratory. Any strain fulfilling two of the following four criteria is worthy of special investigation: (I) atypical morphology; (2) pigment production when incubated in artificial light; (3) Lowenstein-Jensen medium containing 10 µg/ml p-acetamido benzaldehyde thiosemicarbazone.

Although it was found relatively straight forward to recognize pigment production or growth at 25°C.difficulty was often encountered in deciding what constituted atypical morphology, and the well recognized pleomorphism of the tubercle bacillus must inevitably make this so. Furthermore, resistance to 10 µg/ml thiosemicarbazone was encountered rather more frequently than had been expected in an area where the incidence of anonymous mycobacteria was thought to be low (Mitchison, 1962), and for these reasons a more detailed investigation of such strains, and of the patients from whom they were derived, was made.

In this paper are recorded 10 strains of *M. tuberculosis* resistant to thiosemicarbazone, seven of them probably not as a result of previous treatment with this drug. This finding has made us cautious in our interpretation of the results of this part of the screening test for anonymous mycobacteria, and further experience has led us to prefer paranitrobenzoic acid (Marks, 1965; Tsukamuraand Tsukamura, 1964) to thiosemicarbazone for this purpose (Barrie, 1967).

The reemergence of tuberculosis in many countries in different regions of the world is a common public health concern. Rapid and precise diagnosis of each case is necessary for appropriate control of the disease. Isolation, identification and susceptibility testing are essential procedures that should be performed as quickly as possible, so that adequate treatment can be prescribed.

The use of liquid media has been suggested as the most efficient and quickest procedure for the isolation of mycobacteria and susceptibility testing. However, as well as being isolated, these microorganisms should be promptly identified. Although *M. tuberculosis* infection is most common, infection due to mycobacteria other than *M. tuberculosis*, or nontuberculous mycobacteria (NTM), is on the increase in many countries. It is important to establish *M. tuberculosis* infection at an early stage for the establishment of adequate treatment of tuberculosis patients who follow treatment regimens different from patients infected with other mycobacteria.

The BACTEC 460TB (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) method enables differentiation differentiation between the *M. tuberculosis* complex (MTC) and other mycobacteria by means of the NAP test (nitro-acetylamino-hydroxypropriophenone), which produces results in 4–6 days, but uses radioactive media. Other methods, such as molecular probes and high performance liquid chromatography (HPLC), have been proposed in the differentiation of mycobacterial species. These methods, however, require procedures that are technically complex, laborious and costly. Regions that have scarce resources may not be able to perform these procedures and thus require simple but rapid tests.

A method that uses liquid medium in a tube with an indicator for monitoring mycobacterial growth (BD BBL™ MGIT™, Becton Dickinson) was recently introduced. The system is efficient in the rapid isolation and detection of drug resistance in these microorganisms. It is safe, as it does not use radioactive material. The objectives of the present study were to develop a test adding p-nitrobenzoic acid (PNB)

to the medium to evaluate its use in differentiating MTC other mycobacteria. This may reduce the time required for species differentiation.

The ability of mycobacteria to grow in the presence of inhibitory substances in a suitable medium has been widely used in the identification of different species. It has been reported that growth of the MTC is inhibited by PNB 500 µg/ml, whereas NTM are resistant to this concentration. Although a small percentage of these bacteria may be susceptible to the substance, as suggested by Rastogi et al, and Tsukamura et al., the study developed by Martins et al. showed that mycobacterial growth in PNB containing medium may be used as a presumptive test for NTM.

TCH is also used for differentiation within the MTC when performed together with other tests. African and Asian strains, as well as *M. bovis*, are susceptible to TCH, whereas most *M. tuberculosis* isolates from humans were resistant to 5 µg/ml of the compound added to the culture medium (Giampaglia et al, 2005).

3.8.2.5 OTHER IDENTIFICATION TESTS

Arylasulphatase test

The ability of arylsulfatase to break down phenophtalein disulfate into phenopthalein help to differentiate among certain strains of mycobacteria. The organism is grown for two weeks in a medium containing 0.001 M tripotassium phenopthalein disulphate. The liberation of free phenopthalein is detected by the red colour produced on addition of an alkali. *M. tuberculosis* gives a negative test.

Peroxidase test

Isoniazid-sensitive *M. tuberculosis* is peroxidase positive while unclassified mycobacteria are isoniazid resistant strains of *M. tuberculosis* are peroxidase negative.

Susceptibility to Thiophen-2-carboxylic acid hydrazide (TCH)

M. tuberculosis is usually not inhibited by 10ug/ml of TCH; however, many Indian strains of *M. tuberculosis* with low virulence to guinea-pigs are susceptible to TCH. *M. bovis* are usually susceptible.

Susceptibility to pyrazinamide

M. tuberculosis is sensitive to 50µg/ml of pyrazinamide, while *M. bovis* and other mycobacteria are resistant.

Test for iron uptake

A slope of Lowenstein Jensen medium containing 2% ferric ammonium citrate is inoculated with the strain and incubated at 37ºC for three weeks. The appearance of an intense rust colour in the colonies, accompanied by a change of colour in the medium to a pale tan, is interpreted as a positive test. *M. tuberculosis* and other slow- growing mycobacteria give negative test; most rapid growers give positive reactions.

Neutral red test

This test detects the ability of a stain to bind neutral red in an alkaline buffer solution; positive tests are obtained with *M. tuberculosis*, *M. bovis*, *M. avium,* and *M. ulcerans.*

Tween 80 hydrolysis

This test is carried out by suspending the growth from a slope in a mixture of tween 80 buffers neutral red at 37^0C. The reaction is read at 4 hours, 5 days and 10 days. A pink colour indicates hydrolysis of Tween. *M. tuberculosis* does not hydrolyse tween 80. This test is particularly useful in differentiating *M. scrofulaceum* (negative), from *M. gordonae* and *M. flavescens* (positive).

Sugar media

M. tuberculosis does produce any detectable acid in usual sugar media. It utilizes glucose and glycerol which are oxidized completely.

Tellurite reduction test

The enzyme tellurite reductase reduces potassium tellurite to metallic tellurite, which is visualized as a black precipitate. Only *M. avium* complex strains and rapid-growing mycobacteria possess a fast-acting enzyme, which is detected in this test (Tripathy 1981; Baily and Scott 1990).

3.8.3 OTHER TECHNIQUES FOR DIAGNOSIS OF TUBERCULOSIS

3.8.3.1 RADIOGRAPHIC FINDING

Chest X-rays (radiological) can also help in the detection of pulmonary tuberculosis but they do not allow etiological diagnosis. X-ray suggesting tuberculosis include upper-lobe infilterates, cavitary infilterates and hilar or paratracheal adenopathy. In many patients with primary progressive tuberculosis and those with HIV infection, radiographic finding are subtler and can include lower lobe infiltrates or a military pattern. Miliary lesions, which are small granulomas, resemble millet seeds spread throughout the lung fields (Bloom, 1994).

3.8.3.3 IMMUNOLOGICAL DIAGNOSTIC METHODS

a. Antigen detection

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

b. Antibody detection

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

c. ELISPOT test

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

3.8.3.4 TUBERCULOSTEARIC ACID (TSBA) TEST

One easily detected component of *M. tuberculosis* is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography (Brooks et al, 1987).

The presence of tuberculostearic acid in cerebrospinal fluid is thought to be diagnostic for tuberculosis meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis (Savic et al, 1992). However an important concern with pulmonary specimens is that organisms other than *M. tuberculosis* may produce components that will generate a false positive signal (Bloom, 1994).

3.9 PREVENTION AND CONTROL

The prevention of tuberculosis involves either prevention of infection or, if infection has already occurred, elimination of viable populations of organisms within the host. There are relatively two effective methods of preventing clinical tuberculosis-INH prophylaxis and BCG vaccination. These methods should be considered as complementary and non competitive. The BCG vaccination is useless after the patient has been infected with tubercle bacilli, and isoniazid prophylaxis affords no protection to the uninfected person after treatment is stopped.

3.10 TREATMENT

Tuberculosis therapy generally consists of a 6 to 9 months course of isoniazide, rifampicin, streptomycin, thioacetazone pyrazinamide and ethambutol. Besides these main drugs other drugs include cycloserine, ethionamide, and capreomycine. There are three main properties of anti-tuberculosis drugs: bactericidal activity, sterilizing activity and the ability to prevent resistance (Maher et al, 1997).

CHAPTER 4

4. MATERIALS AND METHODOLOGY

4.1 MATERIALS

A complete list of bacteriological media, reagents, chemicals, equipments, glasswares and miscellaneous materials required for this study is given in appendix I.

4.2 METHODOLOGY

4.2.1 STUDY SITE

This study was carried out at National Tuberculosis Center (NTC), Thimi, Bhaktapur from June 2005 to May 2006.

4.2.2 STUDY POPULATION

Sputum smear positive patients: These were new cases of pulmonary tuberculosis visiting NTC who were sputum smear positive in microscopy.

4.2.3 SAMPLE COLLECTION

Sputum is the sample of choice in this study. Among triplicate sputum samples (first, on the spot; second, early morning sample; and third, on the spot) collected at NTC only early morning sample per patient was included in this study. All samples were collected in leak proof, wide mouth, transparent, sterile and stopper plastic container. The patients were given clear instruction about the quality and quantity of samples. Adequate safety precautions were taken during the specimen collection to prevent the spread of infectious organism.

4.2.4 SAMPLE EVALUATION

A good sputum sample consists of recently discharged materials from the bronchial tree, with minimum amount of oral and nasal material. Thus, about 4ml of mucoid or muco-purulent early morning sample was collected and labeled appropriately.

4.2.5 SAMPLE PROCESSING

The collected sputum samples were proceeded for microscopy and culture on the same day of collection.

4.2.5.1 SPUTUM SMEAR MICROSCOPY (WHO, 1998a)

Sputum smear was prepared on a clean, new and unscratched slide at one end with the relevant patient's number. An appropriate portion of the sample was transferred to the slide with the help of the broken end of a wooden stick. The sample was smeared on the slide over an area of approximately 2.0 by 1.0 cm and made it thin enough to be able to read through it. The smear was allowed to air dry for 15 minutes without heating. Thereafter, the smear was heat fixed passing the slide through a flame 3 to 4 times with the smear uppermost and allowed to cool before staining. These entire steps were performed inside a safety cabinet. Among the several methods of determining acid-fast nature of mycobacteria, Ziehl-Neelsen method was included in this study. 1% carbol fuchsin was poured to cover the entire surface of the slides. The slides were heated underneath until vapour start rising. The slides were to stand for 5 minutes. The slides were then rinsed with tap water and excess water was drained off. The slides were decolorized with 3 % acid alcohol for 3 minutes. The slides were rinsed thoroughly with tap water and excess water was drained off. The slides were flooded with 0.3% methylene blue and let to stand for 1 minute. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry. The slides were examined under microscope in 1000x oil immersion.

4.4.5.2 CULTURE (WHO, 1998 b)

Sputum samples after microscopy were mixed with its twice volume of 4% NaOH in a graduated centrifuge tube of 15ml capacity and shaked for several times to digest, then left to stand for 15 minutes at room temperature with occasional shaking. The specimen was centrifuged at 3000 X g for 15 minutes and the sediment was suspended with 15ml distilled water. The tube was again centrifuged at 3000 X g for 15 minutes. The sediment was harvested after discarding the supernatant and was used for culture.

For culture, 0.1ml of concentrated sputum was inoculated into culture tube containing 2% Ogawa medium. These tubes were examined on 7^{th} day for rapid growers and weekly thereafter for slow growers. If any colonies were seen at any stage, acid fastness was determined by smear examination of the growth. Negative report was given when no colonies appeared after observing weekly for 8 weeks.

4.3 NIACIN TEST

Niacin test for the identification of *M. tuberculosis*

Procedure

1. 1ml of sterile water was added to the culture slant. If the growth was confluent, the medium was punctured with pasture pipette to allow contact of water with the medium.
2. The tubes were placed horizontally so that the fluid covers the entire surface of the medium.
3. 30 minutes was allowed for the extraction of niacin. The extraction time may be longer if the culture has few colonies.
4. The slants were raised for 5 minutes to allow the fluid to drain to the bottom.
5. 0.5ml of the fluid extract was removed to a clean screwcap tube.
6. Sequentially added 0.5ml o the 4% aniline solution and 0.5ml of 10% cyanogen bromide solution.
7. The tubes was closed and the solution was observed for the formation of a yellow colour (=positive result) within 5 minutes. The yellow colour appears as a ring at the interface of the two reagents, or if the is shaken, as a yellow column of liquid.
8. 2-3ml of 4% NaOH was added to each tube and discarded.

***Results and interpretation**

Negative: No colour

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

4.4 NITRATE REDUCTION TEST

Nitrate reduction test for identification of *M. tuberculosis*

Procedure

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

    a. 1 drop diluted HCL
    b. 2 drops .02% sulfanilide
    c. 2 drops .01% N-naphthyethylene-diamine

6. Examined immediately for the formation of pink to red colour and compared to the standard.

***Results and interpretation**

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

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

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

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

Only 3+ to 5+ is considered positive.

4.5 CATALASE TEST

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

Procedure

1. With a sterile pipette, aseptically 0.5ml of 0.067/M phosphate buffer, pH 7.0 to 16X125 mm screw cap tubes.
2. Several loopfulls of test cultures was suspended in the buffer solution using sterile loops.
3. The tubes containing the emulsified cultures were placed in a previously heated water bath at 68ºC for 20 minutes. Time and temperature was critical.
4. The tubes were removed from the heat and allowed to cool to room temperature.
5. 0.5ml of freshly prepared Tween-peroxide was added to each tube and caps were replaced loosely.
6. Then it was observed for the formation of bubbles appearing on the surface of the liquid. The tubes were not shaken as Tween 80 may form bubbles when shaken, resulting in false positive results.

The negative tubes were held for 20 minutes before discarding.

Results and interpretation

Positive: Bubbles

Negative: No bubbles

4.6 GROWTH ON MEDIUM CONTAINING P-NITROBENZOIC ACID (PNB)

1. Two slopes of LJ medium containing glycerol and one tube containing p-nitrobenzoic acid was inoculated at a concentration of 500mg/litre.
2. One LJ slope and the PNB slope was incubated at 37ºC in an internally illuminated incubator and examined at 3, 7, 14 and 21 days. When growth was evident on the LJ slope it was examined for pigment. If an internally illuminated incubator was not available, the slopes were removed from the dark incubator as soon as growth was evident, loosened the caps to admit some oxygen and exposed them to daylight (but not direct sunlight) or placed them 1m from the laboratory bench lamp and examined for pigment the following day.
3. The other slope was incubated at 25ºC and examined at 3, 7, 14, and 21 days.

Results and Interpretation

M. tuberculosis does not grow within three days at 37ºC and does not grow at all at 25ºC or on PNB medium. It does not produce yellow or orange pigment in the dark or after exposure to light.

CHAPTER 5

5. RESULT

During the study period a total of 200 culture positive samples were analyzed and 189 of the patient were diagnosed as having PTB by performing Niacin, Nitrate Reduction, 68 degree Heat liable catalase test and growth on PNB containing medium. Out of 200 samples 162 (81%) were sputum smear positive by ZN staining.

In the study group, table 1 shows age and sex wise distribution of total samples. In 200 samples, 142 (71%) were male and 58 (29%) were female. 189 out of 200 samples were confirmed as being infected with *M. tuberculosis* by the above mentioned tests. Among 189 cases 132 (66%) were male and 57 (28.5%) were female. Maximum no. of confirmed TB cases was observed in age group of 21 to 30 (58, 29%). On the basis of age wise distribution no significant difference was seen in TB cases.

5.1 Distribution of total study cases (N=200) By age and sex.

Table 1: Distribution of total cases by age and sex.

Age group (years)	Male		Female		Total	
	No.	%	No.	%	No.	%
0-10	3	1.5	2	1	5	2.5
11-20	9	4.5	3	1.5	12	6
21-30	48	24	15	7.5	63	31.5
31-40	28	14	14	7	40	20
41-50	32	16	10	5	42	21
51-60	14	7	4	2	15	7.5
Above 60	10	5	10	5	23	11.5
Total	**142**	**71**	**58**	**29**	**200**	**100**

In table 2 out of 162 smear positive samples 118 (72.84%) were male and 44 (27.16%) were female. The maximum number of smear positive samples lies in the age group of 21-30 (46, 28.4%) and minimum in the age group of 0-10 (2 female and 1 male).

Table 2: Distribution of total smear positive cases by age and sex

Age Group (Years)	Female		Male		Total	
	No.	%	No.	%	No.	%
0--10	2	1.23	1	0.62	3	1.85
11--20	2	1.23	9	5.56	11	6.79
21--30	11	6.79	35	21.60	46	28.40
31--40	12	7.41	23	14.20	35	21.60
41--50	7	4.32	28	17.28	35	21.60
51--60	4	2.47	14	8.64	18	11.11
Above 60	6	3.70	8	4.94	14	8.64
Total	**44**	**27.16**	**118**	**72.84**	**162**	**100.00**

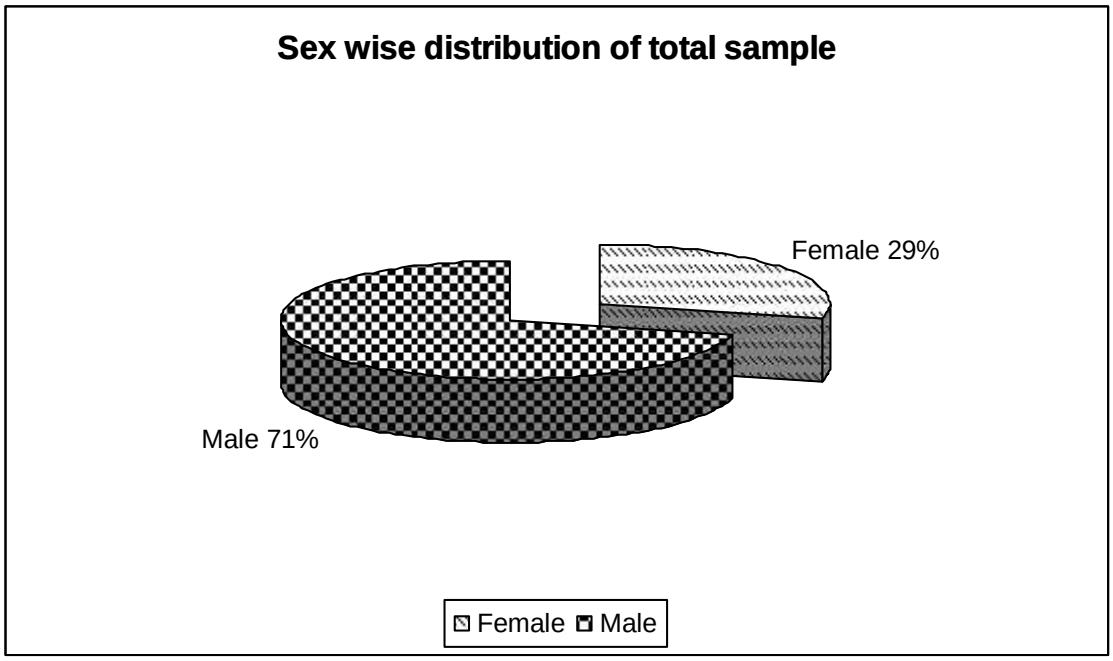

Fig 1 Sex wise distribution of total sample

Considering the distribution of TB among genders, more males have been found to be infected than females, in all age groups (figure 2). Most number of cases occurred in the age group of 21-30 (30.5%) and least in the age group 0-10 (1.5%). Except in age group 0-10 female (1%) and male (0.5%) and in the age group above 60 where male and female cases are equal i.e. 5% in all other age group the number of male exceeds that of female.

Out of 200 samples 142 (71%) were male and 58 (29%) were female. (Fig. 1).

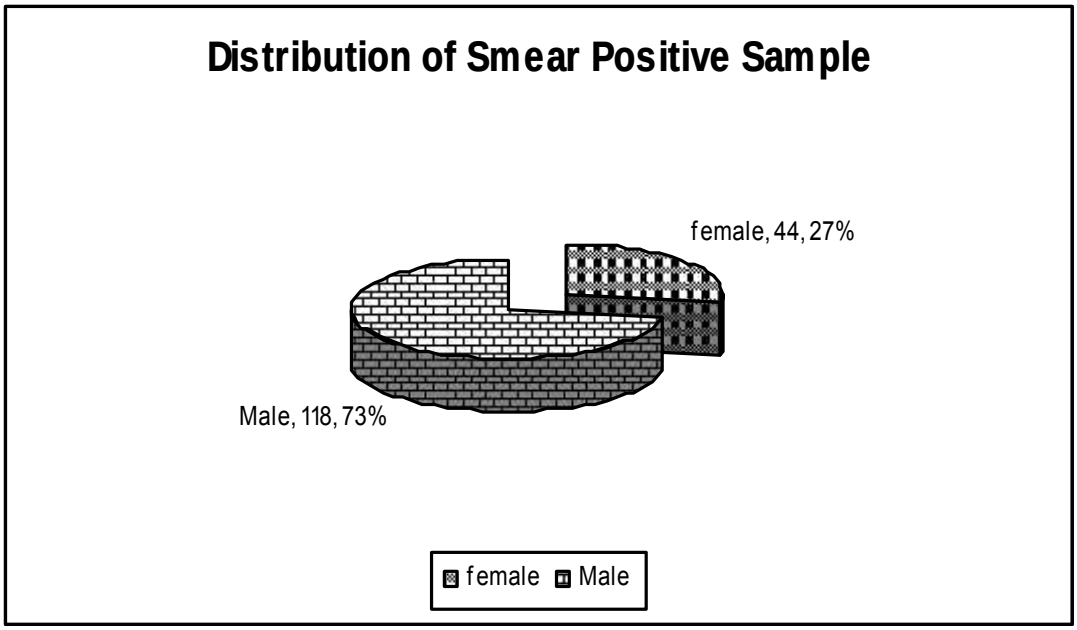

Fig 2 Sex wise distribution of total smear positive sample

Out of 200 sputum samples, 162 (81%) was positive by ZN staining. Out of 162, 118 (73%) were male and 44 (27%) female.

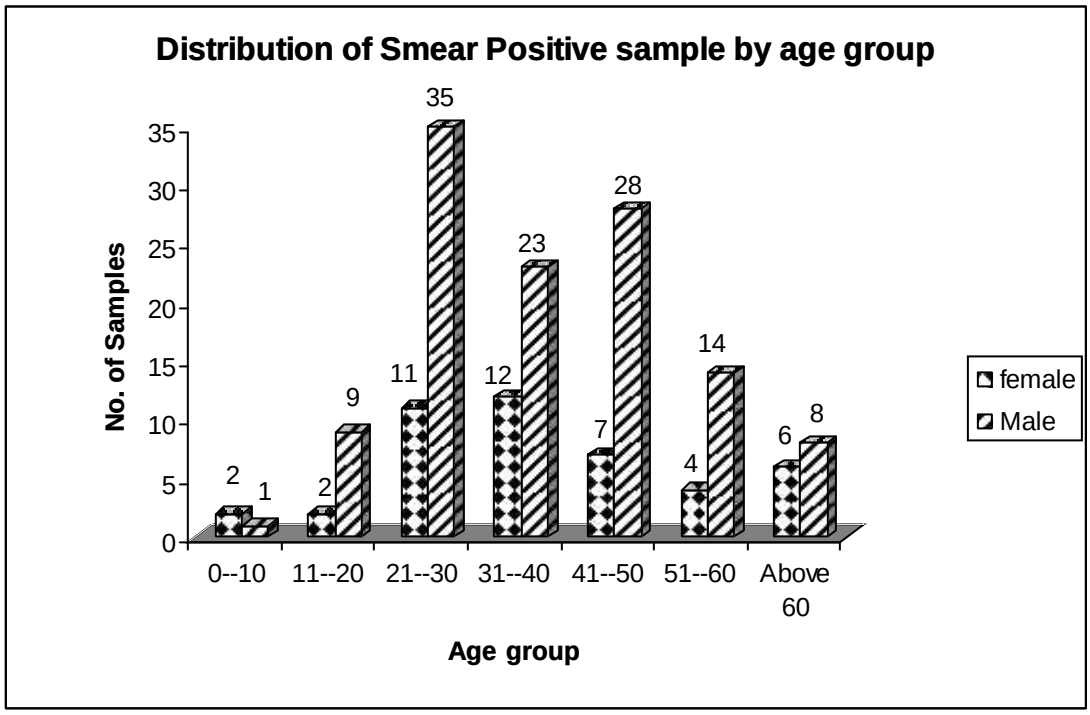

Fig 3 Bar diagram depicting age wise sex distribution in total smear positive samples

In the distribution of sputum smear positive samples the highest number was found in the age group of 21-30 (46) and lowest was in the age group of 0-10 (3). In all age groups except 0-10 the number of male smear positive sample is greater than females.

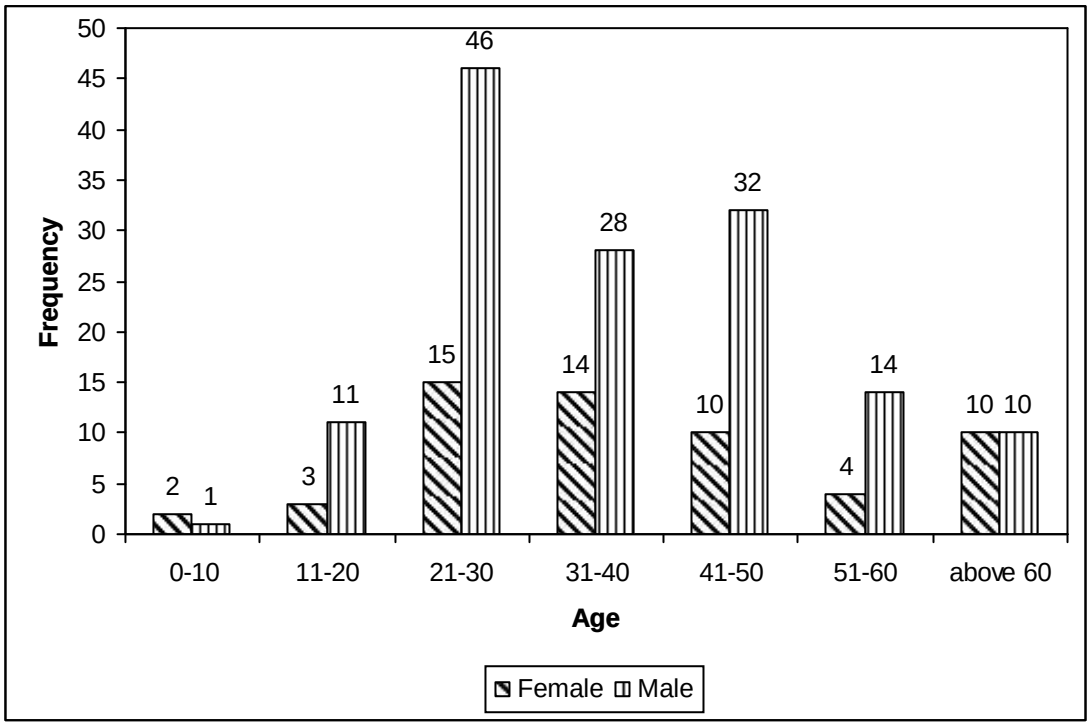

Fig 4 Bar diagram depicting age wise sex distribution in total samples

The present study conducted different biochemical tests for the diagnosis of PTB. The different tests were Niacin test, Nitrate reduction test, Heat labile catalase test and growth on PNB containing medium. 95% were positive for Niacin test, 94.5% were positive for Nitrate reduction test, 97% were negative for heat labile catalase test and no growth was seen on 93.5% samples.

Out of 200 samples in all age groups the number of males is greater than females except in age group 0-10 (2 female and 1 male) and the age group above 60 (10 male and female each).

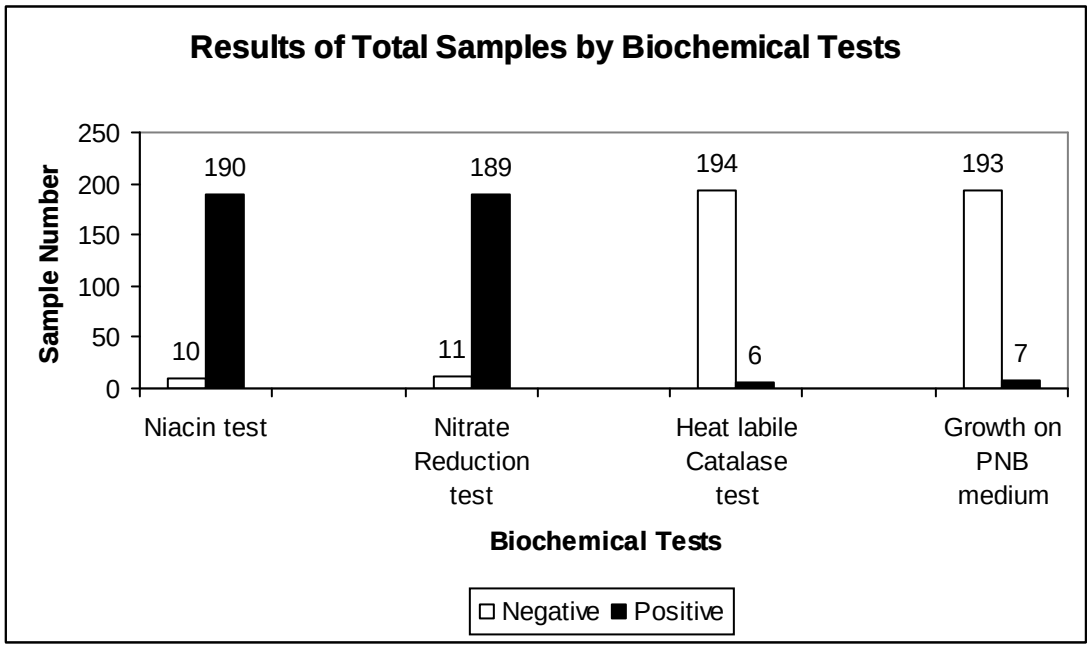

Fig 5 Result of total samples by Biochemical tests.

In this study out of 200 samples 1% were Niacin negative, Nitrate reduction negative and heat labile catalase negative and growth on PNB. 1% negative for first three tests and no growth on PNB, 2.5% negative for the first two, positive for third and growth on fourth. 94.5% were positive for the first two tests and negative for last two which confirmed these samples to be *M. tuberculosis* and rest MOTT.

Table 3: Comparative results of biochemical tests of total samples.

Niacin test	Nitrate Reduction test	Heat labile Catalase test	Growth on PNB medium	Sample Number	Percent
Negative	Negative	Negative	Growth	2	1.00%
Negative	Negative	Negative	No Growth	2	1.00%
Negative	Negative	Positive	Growth	5	2.50%
Negative	Negative	Positive	No Growth	1	0.50%
Positive	Negative	Negative	No Growth	1	0.50%
Positive	Positive	Negative	No Growth	189	94.50%
			Total	**200**	**100.00%**

Out of 200 samples, 189 (94.4%) samples tested positive for Niacin test and Nitrate reduction test, negative for heat labile catalase test and no growth on PNB containing medium. Thus 189 samples can be confirmed as *M. tuberculosis*. The rest are MOTT and their specific identification requires different biochemical tests and if possible PCR.

CHAPTER 6

6. DISCUSSION

Tuberculosis is one of the major public health problems in the third world countries. Nepal is facing with an estimated 45% of the total population being infected, out of which 60% of the adult population being infected with TB. Every year 40,000 people develop active pulmonary tuberculosis cases (PTB). Although a presumptive diagnosis of Tuberculosis is done by sputum smear microscopy and culture on media the definite diagnosis is not possible without the biochemical test or any other rapid identification tests like NAA. So the main objective of this study is to identify whether the culture isolates are *M. tuberculosis* or MOTT by performing biochemical tests. (WHO,1998).

During this study, 200 culture isolates from the sputum samples of patients were subcultured on LJ media and observed for growth on day 4. If no growth was seen it was further incubated for 28 days. Then the growth was subjected for the biochemical testing and growth on PNB containing medium. 189 (94.5%) out of 200 were confirmed as *M. tuberculosis* as they were positive for Niacin and Nitrate reduction test , negative for heat labile catalase test and there was no growth on PNB containing medium. 11 (5.5%) were confirmed as MOTT. The definite identification of MOTT required an array of tests which was out of scope of this study.

Niacin test one of the most important tests for the identification of *M. tuberculosis*. Niacin (nicotinic acid) plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all mycobacteria. Although all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests.

Cultures grown on egg medium yield the most consistent results in the niacin test and LJ medium is therefore recommended. A culture must be at least three to four weeks old and must have sufficient growth of more than 50 colonies. Because *M. tuberculosis* excretes niacin into the growth medium, cultures with confluent growth may give a false-negative niacin reaction because the extracting fluid cannot come in contact with the culture medium. When this occurs, expose the underlying medium surface by either scraping away or puncturing through some of the culture growth.

Aeration of cultures intended for niacin testing is very important. Caps should be loose on slants throughout the entire incubation period and special Cap-o-Test stoppers are recommended.

The development of a reagent-impregnated paper strip test for niacin is based on the formation of cyanogens chloride by the reaction of chloramines-T and potassium thiocyanate in the presence of citric acid. Rupture of pyridine ring of niacin by cyanogens chloride yields γ-carboxy glutanic aldehyde and coupling with a primary amine produces a yellow color. In the development of a yellow color, the formation of a a Schiff base as described by Feigl appears to be the reaction involved in this test system; the strip differs from other procedures in the use of cyanogen chloride instead of cyanogen bromide. When compared to the aniline test in the identification of mycobacteria, the strip method gave equal or superior results. Of 378 cultures tested, 370 (98%) gave identical results by both methods. The remaining eight cultures were initially strip-positive and aniline-negative. Retesting after additional incubation showed that seven of eight were in fact niacin-positive. The remaining organism was not readable by the strip method due heavy pigmentation of the culture. This was not considered significant, since the utility of the niacin is to differentiate among non-pigmented cultures (Young et al, 1970).

The postulated reactions are shown as follows:

Fig 6 Proposed chemical reactions occurring in the formation of yellow color. (1) Production of cyanogen chloride, (2) hydrolysis of niacin, and (3) formation of Schiff base.

Niacin test by aniline and cyanogens bromide although very useful in the identification of *M. tuberculosis* is risky due the toxic effects of ethanolic anline and cyanogens bromide. Therefore this test should be performed with a strip which is quick, easy, reliable and safe. During this study aniline and cyanogen bromide method had to be used due to the unavailability of Niacin test strips in the local market. Even though, if it were available in the market it would have been unaffordable due to its high cost.

The nitrate reduction test is one of a battery of biochemical tests used to identify members of the genus *Mycobacterium* based on the presence of the enzyme nitrate reductase. The end products of nitrate reduction are many and depend upon the bacterial species. The most common end product is molecular nitrogen by way of nitrite reduction. Reduction is evidenced by either the presence of a catabolic end product or the absence of nitrate in the medium. The reduction of nitrate is denoted by a color development when nitrite reacts with the three reagents: hydrochloric acid, sulfanilic acid and naphthyl ethylenediamine dihydrochloride. The resulting red color is due to the formation of a diazonium compound. When confirming a negative test with zinc dust, the reduction of the diazonium salt by the zinc in the presence of acetic acid produces a colored compound, arhydrazine (ATCC, 2005).

Negative results (nitrate not reduced) were confirmed by adding a pinch of zinc dust to the tube. The development of a red color following the addition of Zinc dust confirms

the negative result; nitrate was not reduced initially. If no color change occurred upon addition of zinc dust, the result was positive; nitrate was reduced beyond nitrite to a colorless compound. In such cases, the test should be repeated to confirm the observation.

Limitations

1. A positive test for nitrate reduction may flash instantly or quickly fade.
2. The ability of acid-fast bacilli to reduce nitrate is influenced by the age of the colonies, temperature, pH, and enzyme inhibitors. Rapid growers can be tested within 2 weeks; slow growers should be tested after 3 to 4 weeks of luxuriant growth.
3. For best results, a heavy inoculum of organisms should be used.
4. False-negative results may occur if the culture was too old or the reagents were not active or added in the wrong sequence (ATCC, 2005).

In this study, there were 189 (94.5%) positives cases for nitrate reduction and rest negative. Although this test showed a high percentage of positive cases it is not without problems. Reagents used in the conventional test have a short life and must be prepared in-house. The test can be difficult to read because the positive reaction color can flash instantly or quickly fade. Commercially available paper strip reagents have a longer shelf life, but they usually are positive for mycobacteria with strong nitrate reductase activity.

In this study 194 (97%) samples were negative for 68ºC heat labile catalase test and 6 (3%) were positive for the test. The negative results confirm that the samples were *M. tuberculosis* and rest MOTT. Of the biochemical four tests this test has given the highest results in confirming *M. tuberculosis*.

This test is used to differentiate those bacteria that produce catalase from non-catalase producing bacteria. Catalase is an intracellular soluble enzyme capable of splitting H_2O_2

into water and oxygen. The oxygen bubbles into the reaction mixture indicate catalase activity. Catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Reduced flavoprotein reacts directly with gaseous oxygen to form H_2O_2 which is an oxidative end product of the aerobic breakdown of sugars. H_2O_2 thus formed is toxic to bacteria, resulting in their death. The enzyme catalase decomposes H_2O_2 into water and oxygen.

In heat labile catalase test the catalase activity is lost due to heat and thus *M. tuberculosis* gives negative result.

Two classes of catalase have been recognized among the mycobacteria. M-catalase retains its activity after 1 minute exposure to 68ºC, but is inactivated by aminotrazole; T-catalase is inactivated by heat, but resists aminotriazole. Both classes retain their activity after precipitation by specific antibody, and many species of mycobacteria produce both catalases. T-catalase is produced by virtually all pathogenic, slowly growing mycobacteria. T-catalase is a better enzyme for identification purposes than is M-catalase, since it is found in all pathogenic species of cultivable, slowly growing mycobacteria, with the occasional exception among some strains of *M. tuberculosis* that have mutated to high isoniazid resistance. M-catalase producers are species of slow growers, *M. asiaticum*, *M. gordonae*, *M. scrofiulaceum*, *M. simiae*, and *M. szulgai*. Examples of T-catalase producers are *M.avium*, *M. bovis*, *M. intracellulare*, *M. tuberculosis* (Wayne and Gilbert, 1987).

In this study there was no growth observed on 193 (96.5%) samples and growth was observed in 7 (3.5%) samples.

Mycobacterial growth in media to which inhibitory substances are added has been used in species identification. Growth of MTC id inhibited by PNB, whereas NTM are resistant. Thiophene-2-carboxylic acid hydrazide (TCH) is useful in the differentiation of MTC when performed together with other tests.

The ability of mycobacteria to grow in the presence of inhibitory substances in a suitable medium has been widely used in the identification of different species. It has been reported that growth of the Mycobacterium tuberculosis complex (MTC) is inhibited by PNB 500µg/ml, whereas non-tuberculous mycobacteria (NTM) are resistant to this concentration. Although a small percentage of these bacteria may be susceptible to the substance, as suggested by Rastogi et al and Tsukamura et al., the study developed by Martins et al showed that mycobacterial growth in PNB containing medium may be used as a presumptive test for NTM.

A method that uses liquid medium in a tube with an indicator for monitoring mycobacterial growth (BD BBL™ MGIT™, Becton Dickinson) was recently introduced. The system is efficient in the rapid isolation and detection of drug resistance in these microorganisms. It is safe, as it does not use radioactive material. The objectives of the present study were to develop a test adding para-nitrobenzoic acid (PNB) to the medium to evaluate its use in differentiating MTC from other mycobacteria. This may reduce the time required for species differentiation.

To establish that PNB concentration would also work in MGIT, we conducted preliminary experiments testing different concentrations of PNB (125 µg/ml, 250 µg/ml and 500 µg/ml) in BBL-MGIT media. The results showed that all 17 *M. tuberculosis* strains were susceptible to PNB at concentrations of 250 µg/ml and 500 µg/ml, and that four strains were resistant to PNB at 125 µg/ml but susceptible at 500 µg/ml. Based on these results it was concluded that the optimal concentration was 500 µg/ml.

Species differentiation of MTC with the MGIT/TCH method was similar to that observed with the conventional LJ/TCH method test, nitrate reduction and catalase production, but these tests are time consuming. The PNB test, which gave the best result, requires two additional MGIT tubes for a definitive identification. This test has many advantages: it usually takes 5 instead of 30 days using conventional biochemical

methods, it will improve the overall protocol of isolation, identification and susceptibility testing using the MGIT system, and the cost of combined niacin strips, nitrate and catalase reagents is similar to that of the two extra tubes of test, nitrate reduction and catalase production, but these tests are time consuming. The PNB test, which gave the best result, requires two additional MGIT tubes for a definitive identification. This test has many advantages: it usually takes 5 instead of 30 days using conventional biochemical methods, it will improve the overall protocol of isolation, identification and susceptibility testing using the MGIT system, and the cost of combined niacin strips, nitrate and catalase reagents is similar to that of the two extra tubes of MGIT (Giampaglia et al, 2005).

Out of 200 samples 189 showed positive reaction for Niacin test and Nitrate Reduction test; Negative reaction to heat labile catalase test and no growth on PNB containing medium. Thus 189 samples could be confirmed as *M. tuberculosis* and rest as MOTT.

The purpose of the investigation was to determine the differences in the metabolic processes between the human and bovine strains of *M. tuberculosis* which result in the accumulation of nicotinic acid only by the human strains. It will be shown that the source of nicotinic acid which accumulates in the culture medium is nicotinamide adenine dinucleotide (NAD) and that a major difference between the two varieties of *M. tuberculosis* is the relatively high activity of NAD glycohydrolase in human strains as compared with negligible activity of this enzyme in bovine strains (Kasarov and Moat, 1971).

The development of a reagent-impregnated paper strip test for niacin is described. The test system is based on the formation of cyanogen chloride by the reaction of chloramine-T and potassium thiocyanate in the presence of citric acid. Rupture of the pyridine ring of niacin by cyanogen chloride yields y-carboxy glutaconic aldehyde and coupling with a primary aromatic amine produces a yellow color. Sensitivity to niacin, both in known solutions and from extracts of 378 clinical mycobacteria isolates,

equalled and exceeded that of other methods for detection of niacin. Correlation with other tests for mycobacterial niacin was excellent (Young et al, 1970).

In the development of a yellow color, the formation of a Schiff base as described by Feigl appears to be the reaction involved in this test system; the strip test differs from other procedures bromide. The productions of cyanogen chloride from acidified KSCN and chloramine-T have been described by Kraus and Krausova and by Kilburn and Kubica. Color development is linear within the range tested, and response slopes of the two systems are not significantly different.

When compared to the aniline test in the identification of mycobacteria, the strip method gave equal or superior results. Of 378 cultures tested, 370 (98 %) gave identical results by both methods. The remaining eight cultures were initially strip positive and aniline-negative. Retesting after additional incubation showed that seven of the eight were in fact niacin-positive. The remaining organism was not readable by the strip method due to heavy pigmentation of the culture. This was not considered significant, since the utility of the niacin test is to differentiate among non pigmented cultures (Young et al, 1970).

For a biochemical test to be useful for identifying bacteria, it should exhibit a high degree of sensitivity, specificity, and ease of interpretation. Additionally, reagent preparation should be as convenient as possible. The conventional nitrate reductase test has been shown to be reliable and specific, but difficulties with the procedure have been observed. Conventional reagents have a short shelf life which requires frequent preparation. If the reaction is not watched carefully by the microbiologist performing the test, the color reaction can be missed. When a number of tests are being carried out, fading of the positive reaction to a negative reaction can cause confusion when the technologist reviews the results. Use of the crystalline reagent eliminated these technical difficulties. Preparation was easy, the shelf life was found to be at least 6

months, and reading the test was easier. Positive color reactions were always stable which, was not the case when the conventional liquid reagents were used. For example, all of the *M. szulgai* isolates and one half of the *M. terrae* isolates were observed flashing or fading in color when the liquid reagents were used (Warren et al, 1983).

During 1963-64 the use of thiosemicarbazone (T.S.C.) was helpful in detecting seven strains of anonymous mycobacteria, but in a slightly larger number 'false positive' results were obtained. As nine of these 10 strains showed resistance streptomycin, P.A.S., or isoniazid the possibility was entertained that other drugs might have been used in the patient's treatment. Particularly relevant here is the role of ethionamide, in view of the complex cross-resistance which exists between this drug and thiosemicarbazone. The factors involved in this cross-resistance have been discussed at length by Rist (1964), but it has been our experience that, at the critical levels' employed, this cross-resistance is not complete. Five of the 10 thiosemicarbazone resistant strains were sensitive to ethionamide, and in a number of instances they have recorded ethionamide resistance in strains susceptible to thiosemicarbazone.

Furthermore, it is seldom possible to decide in the laboratory whether a strain's resistance to thiosemicarbazone is innate and probably indicative of ananonymous mycobacterium, or acquired as a result of therapy with thiosemicarbazone (or allied compound)or even ethionamide. This is undoubtedly a significant handicap in its use as a screening test.

Encouraged by the fact that susceptibility to para-nitrobenzoic acid correctly classified the 10 strains whose status had been in doubt we incorporated this test in their screening procedure as a substitute for thiosemicarbazone. A further 1,100 strains have now been tested; eight were resistant to para-nitrobenzoic acid, and these all proved, on further tests, to be anonymous strains. No false positive results were obtained. An additional advantage is that strains of *M. kansasii* have proved resistant to para-nitrobenzoic acid, whereas they were sensitive to thiosemicarbazone (Barrie, 1967)

The diseases produced by species of the genus *Mycobacterium* are important causes of morbidity and mortality in the world; they have increased due to HIV infections, with the involvement mainly of *M. tuberculosis* and *M. avium* complexes. The identification of mycobacteria to the species level is important because of the clinical significance; some species are pathogenic while others are not. Knowledge of species is also critical in order to provide adequate patient management because specific antimycobacterial drugs are required against different pathogenic mycobacteria species.

The conventional methods for the identification of mycobacteria, currently used at Instituto de Diagnósticoy Referencia Epidemiológicos (InDRE) in Mexico City, are based on culture and biochemical tests, they require several weeks for adequate growth, and sometimes, accurate identification is not possible. Difficulties such as lack of adequate reproducibility, the variability of phenotypes, and the fact that phenotype information is limited to common species, may lead to ambiguous or erroneous results. Alternative techniques have been established, such as thin layer chromatography, gas-liquid chromatography, high-performance liquid chromatography (HPLC), and molecular techniques based on hybridization, amplification, or sequencing of nucleic acids, but in developing countries they are generally limited to research laboratories (Baretto et al, 2000).

Tests to detect and identify *Mycobacterium tuberculosis* complex (MTB) and other mycobacteria now include both conventional and alternative methods. The conventional tests detect visible properties of the organism, such as acid-fastness, colony morphology, and biochemical reactions. By contrast, the newer alternative methods directly detect the genetic composition or other components and products of the organism.

If it is desirable to identify *M. tuberculosis* complex prior to processing for drug susceptibility testing, the culture, if affluently growing, can be subjected to biochemical

tests, i.e., niacin, nitrate reduction, and 68°C labile catalase tests that permit identification of *M. tuberculosis* and *M. bovis.* It should be noted, however, that some strains of *M. tuberculosis* and *M. bovis* are niacin negative. Cultures with too scanty growth for biochemical tests are tested against the anti-tuberculosis drugs isoniazid, rifampicin, streptomycin, and ethambutol plus para-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. Almost all strains of *M. tuberculosis* complex are susceptible to para-nitrobenzoic acid and *M. bovis* is susceptible to both para nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. However, identification of *M. bovis* must be ensured by niacin and nitrate reduction tests. In a reas of the world where *M. africanum* is prevalent, its proper identification is difficult and not recommended if pyrazinamide susceptibility is not being tested.

If it is considered to be more economical to perform identification and drug susceptibility testing simultaneously, the cultures grown on para-nitrobenzoic acid have to be submitted to biochemical tests (IUATLD, 1998).

The association of direct sputum smear microscopy by ZN staining is greater in 21-30 age group and greater in male than female is statistically significant. (Appendix V, xix). Considering the gender in smear positive cases more males were found than females (figure 2). This does not however reflect an increase in occurrence of TB in males, since in this study the attendance of female was lower than female (Table 2). Similar results were obtained during the identification of *M. tuberculosis* by four biochemical tests. (figure 5 and table 2). In the overall study the attendance of males was greater than females.

CHAPTER 7

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

During the study period of June 2005 to May 2006 a total of 200 clinically suspected sputum samples were examined by ZN staining and cultured in Ogawa medium. Primary culture samples were subcultured in the LJ medium. The subcultures were observed for their cultural characters for 4 weeks and then subjected for biochemical tests for their confirmation as *M. tuberculosis.* From the 200 subcultures Niacin, Nitrate reduction, 68ºC labile catalase test and growth on PNB containing medium was performed. Out 200 samples 190 (95%), 189 (94.5%), 6 (3%) and 7 (3.5%) were positive for Niacin, Nitrate reduction, 68ºC labile catalase and growth on PNB containing medium and 10 (5%), 11 (5.5), 194 (97%) and 193 (96.5%) were negative for the respective tests.

Out of 200 samples 189 (94.5%) were positive for Niacin and Nitrate reduction and negative for 68ºC labile catalase and growth on PNB containing medium which confirms that they were *M. tuberculosis.* 11 (5.5%) were confirmed as MOTT which could not be identified to the species level.

7.2 Recommendations

Based on the finding and experience of this study, the following recommendations have been made.

1. Niacin test is one of the most important tests for the identification of *M. tuberculosis*. It is better to perform this test by paper strip method as Aniline is oncogenic and penetrates through the skin; Cyanogen bromide is a severe lacrimator and toxic if inhaled. Cyanogen bromide is also oncogenic. Thus there is a high risk involved.
2. In Nitrate reduction test performed by this conventional method problems like fading of color, instant flashing were encountered in the positive reaction thus

making it difficult to read. Reagents used in this test have a short shelf life and must be prepared in-house. The new reagent described by Lampe consists of one part sulfanilic acid, one part N-(onenaphthyl) ethylenediamine dihydrochloride, and 10 parts L-(+)-tartaric acid is recommended for this test.

3. Neither the conventional methods nor the newer alternative methods alone satisfy all requirements of definitive identification, rapid results, and cost effectiveness. Also, even if *M. tuberculosis* is identified by an alternative method such as nucleic acid amplification, the organism must still be cultured for susceptibility testing. For these reasons, a combination of alternate and conventional method is recommended for the proper management of the disease.
4. A simple, low-cost test using growth inhibitors like PNB may be incorporated into a modern, safe and quick methodology enabling differentiation of MTC and NTM.

CHAPTER 8

8. REFERENCES

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