CHAPTER-I

1. INTRODUCTION

Tuberculosis is a specific contagious infectious disease caused by *Mycobacterium tuberculosis.* Inspite of the availability of highly effective drugs and vaccine and all other advances in knowledge of this disease to make it preventable and curable, tuberculosis still remains one of the major health problems faced by mankind. The mycobacterial diseases result in significant amounts of morbidity and mortality worldwide (Park, 2000). The WHO declared a state of global emergency for tuberculosis in 1993 due to the steady increase of the disease (WHO, 1993).

Presently, about one third of the world's population is infected with tuberculosis. It is has been estimated that there are about 8 million new cases every year and 3 million deaths occurring worldwide (Dye *et al.*, 1999). The WHO estimated that within the year 2002 and 2020, nearly one billion people will be newly infected, 150 million people will get ill, and 36 million will die from TB if effective control measures are not strengthened (Johansen, 2006); and a total of more than 200 million new cases will arise over the next two decades (Dye C., 2000).

The WHO estimated that 8.8 million case incidences and 1.6 million deaths occurred from TB in 2005 globally. The largest number of new TB cases in 2005 occurred in South-East Asia Region, which accounted for 35% of incident cases (WHO, 2007) and SAARC region accounts for 27.4 percent of TB cases globally with nearly 2.4 million new cases reported every year.

According to the WHO, Nepal is 27th highest TB burden country in the world, with estimated 1.8% (WHO, 2001). Tuberculosis case are scattered all over the country but majority of cases are in rural areas. According to NTP, over 80,000 have already developed TB (NTP, 2002) and 5,000-7,000 people continue to die every year from this disease (NTP, 2006).

The diagnosis of tuberculosis infection is vital both clinically and epidemiologically (Barez *et al.*,1995). Early diagnosis, effective treatment and successful cessation of transmission are major strategies in the control of TB (NTC, 2002/2003). Tuberculosis is diagnosed by clinical symptoms, chest X-ray and sputum smear microscopy. It is confirmed by *in vitro* culture methods and causative organisms identified using

differential *in vitro* tests (WHO, 1998b). Diagnosis by means of radiographic examination is unreliable because other chest diseases resemble tuberculosis on X-ray (Kent and Kubica, 1985).

The core stone of the laboratory diagnosis of TB is the direct microscopic examination of appropriately stained sputum specimens for tubercule bacilli (WHO, 1998a). The detection of AFB in sputum establishes a presumptive diagnosis of TB and is crucial to guide treatment, limit person to person spread and assess the degree of the activity of the disease (Behr *et al*., 1999). Though sputum examination by microscopy is relatively quick, easy and inexpensive, this method has low sensitivity (40-75%) and requires organism load of 5000-10000 bacteria per ml for direct microscopy to be positive; and only a portion of TB patients harbor large enough numbers of organisms to be detected in this way. Furthermore, it is not definitive in terms of species identification and differential diagnosis (Woods and Washington, 1987). However, because acid-fast smear results are available rapidly, they are used to manage patient care and to make public health decision and useful only as a presumptive screening test (Kent and Kubica, 1985).

The cultural method is considered to be the gold standard for confirmatory diagnosis of TB (WHO, 2006) because of its high degree of sensitivity (80% to 93%) and specificity (98%) (ATS-CDC, 2000). Examination by bacteriological culture provides definitive diagnosis of tuberculosis. Depending on the decontamination method and type of culture method used, as few as 10 viable tubercule bacilli can be detected. However, the usual microbiological techniques of plating clinical material on selective or differential culture media cannot be applied to tuberculosis bacteriology (WHO, 1998b). Compared to other bacteria which typically reproduce within minutes, *M. tuberculosis* proliferates extremely slowly (generation time 18-24). The entire process requires 4-8 weeks for species identification (Chakraborty, 2003). Furthermore, the growth requirements are such that it will not grow by primary isolation on simple chemically defined media. The only media which allows abundant growth of *M. tuberculosis* are egg-enriched media containing glycerol and asparagines; and agar or liquid medium supplemented with bovine albumin serum. The most widely used solid media are MLJ and MOG media. The liquid culture media give result within 5 to 7 days but are extremely expensive and positive results require further testing to distinguish between tubercle bacilli and other mycobacteria (WHO, 1998b). Culture is essential in situations like drug susceptibility testing, for change of drug regimen in resistant cases, epidemiological surveys, mass

case-finding programmes, and identification of mycobacterial species and assessment of tuberculosis programmes (Bhargava *et al.*, 2007).

Most of the culture and sensitivity testing facilities for tuberculosis diagnosis in developing countries are centralized only at central level. In Nepal these services are available at NTC, Bhaktapur and GENETUP, Kalimati only. According to WHO, 380 smear testing facilities are active in Nepal (WHO, 2007). Most of these laboratories are only able to conduct sputum smear microscopy and located at provincial and district hospitals. Most of the suspected cases are referred to central level for cultural confirmation or are treated on the basis of physical symptoms and smear microscopy. For a country like Nepal, where most of the case incidences occur in rural region, it is desirable that cultural facilities be available in these areas also.

The MLJ has been recommended by International Union against Tuberculosis and Lung Disease (IUATLD) to be used for the routine culture of *M. tuberculosis* because of its high predictive value than Ogawa (Hans *et al.*, 1998). In many laboratories Ogawa media is commonly used because it is cheaper and gives less contamination and greater positive result. The present study evaluates the improvement of culture positive results by optimization of monopotassium phosphate buffer percentage in MLJ. MOG contains 2% monopotassium phosphate buffer while MLJ contains only 0.4% monopotassium phosphate buffer. The results obtained by increasing monopotassium phosphate buffer level in MLJ from 0.4% to 2% (BLJ) are compared with that of MLJ and MOG under the same conditions.

Hence, the present study aimed to investigate the use of modified culture medium to increase sensitivity of diagnosis which might be helpful for better diagnosis and consequently better management of pulmonary tuberculosis.

CHAPTER-II

2. OBJECTIVES

2.1 General Objective

To compare the growth of *Mycobacterium tuberculosis* bacilli on Lowenstein-Jensen media (MLJ), Modified Ogawa (MOG) media, and newly optimized 2%phosphate buffer added Lowenstein-Jensen (BLJ) media by decontaminating them with 4% NaOH (Petroff's method) and Nekal method.

2.2 Specific Objectives

- Evaluate and access the recovery of *Mycobacterium tuberculosis* in reformulated Lowenstein-Jensen media: BLJ.
- To compare the growth pattern of *Mycobacterium tuberculosis* on BLJ with that of MLJ and MOG.
- To compare two decontamination techniques i.e. 4% NaOH (Petroff's method) and Nekal method.

CHAPTER-III

3. LITERATURE REVIEW

Tuberculosis poses an enormous health problem in many parts of the world, particularly in low-income countries. Progress has been made with the implementation of effective control strategies; many countries, however, still fail to sufficiently recognize the importance and priority of good tuberculosis control. Tuberculosis bacteriology for the detection of sources of infection, the diagnosis of clinical suspects, and the follow-up of the effect and results of treatment, are essential components of tuberculosis control (Hans *et al.*, 1998).

3.1 Epidemiology

Tuberculosis remains one of the deadliest diseases in the world. Each year more than 8 million new cases of tuberculosis occur and approximately 3 million persons die from the disease (ATS-CDC, 2000); of them, about one million are from the SAARC region (WHO fact sheet, 2006). The global incidence rate of tuberculosis is growing at approximately 1.1% per year. Developing countries suffer the burnt of tuberculosis epidemic to a greater extent (Ahlburg, 2000). Death from tuberculosis comprises 25% of all avoidable deaths in developing countries. Nearly 95% of all tuberculosis cases and 98% of deaths due to tuberculosis are in developing countries and 75% of deaths due to tuberculosis cases are in the economically productive age group (WHO fact sheet, 2002).

In 2005, estimated per capita TB incidence was stable or falling in all six WHO regions. However, the slow decline in incidence rates per capita is offset by population growth. Consequently, the number of new cases arising each year is still increasing globally and in the WHO regions of Africa, the Eastern Mediterranean and South-East Asia (WHO fact sheet, 2007).

The WHO estimates that 8.8 million case incidences, including 3.9 million new cases and 1.6 million deaths occurred from TB in 2005. The largest number of new TB cases in 2005 occurred in South-East Asia Region, which accounted for 35% of incident cases globally, followed by Pacific Region (25%) and African Region (23%) (WHO, 2007). However, the estimated incidence per capita in sub-Saharan Africa is nearly twice that

of the South-East Asia Region, at nearly 350 cases per 1,00,000 population (WHO fact sheet, 2007). Both the highest number of deaths and the highest mortality per capita are in the WHO African Region, where HIV has led to rapid growth of the TB epidemic and increase the likelihood of dying from TB (WHO fact sheet, 2007).

The south-East Asian Region includes Bangladesh, Bhutan, India, Korea, Indonesia, Maldives, Mynmar, Nepal, Srilanka, Thailand and Timor-Leste. Out of 22 High Burden countries (HBC) ranked by estimated number of incident cases, India, China, Indonesia, Nigeria, Bangladesh and Pakistan are ranked from 1st position to 6th position respectively (WHO, 2007). India caries the greatest burden of all TB cases. The SAARC region accounts for 27.4 percent of TB cases globally with nearly 2.4 million new cases reported every year. Of these, 1.1 million are capable of communicating the disease to others. Although the SAARC Tuberculosis Centre is located in Kathmandu, only 87 percent of the infected population has access to TB treatment in Nepal (Indo-Asian News Service, 2005).

Nepal has an elevated annual risk of TB infection. Over 80,000 have already developed TB (NTP, 2002). According to the WHO, Nepal is 27th highest TB burden country in the world, with estimated 1.8% incidence (WHO, 2001). Tuberculosis case are scattered all over the country but majority of cases are in rural areas where more than 90% of population reside (Amatya, 1992). Each year, 44,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. It is estimated that 5,000-7,000 people die from TB every year (NTP, 2006). Introduction of treatment by Directly Observed Treatment Short-course (DOTS) has reduced the number of deaths caused by tuberculosis (NTP, 2004). In Nepal, DOTS strategy has been implemented since 1996 which have already proven its efficacy in Nepal and will have a profound impact on mortality and morbidity (NTC 2001/2002). AIDS and HIV contribute to the rising incidence of TB, as they reduce the immunity of a patient and make him vulnerable to TB strains (Indo-Asian News Service, 2005). It accounts for about 13% of AIDS deaths worldwide.

The threat of TB to mankind is no longer restricted to developing nations since it has now returned to industrialized countries due to AIDS epidemic and the emergence of Multidrug resistant (MDR) strains (Mehra and Rajalingam, 2001). HIV and TB form a lethal combination, each speeding the other's progress. HIV weakens the immune system. Someone who is HIV-positive and infected with TB bacilli is many times more

likely to become sick with TB than someone infected with TB bacilli who is HIV-negative. TB is a leading cause of death among people who are HIV-positive patients (WHO fact sheet, 2007). To cope with this problem, WHO has introduced programmes to implement collaborative TB/HIV activities from past five years, which includes a national plan for collaborative TB/HIV activities with HIV counseling and testing for all TB patients through NTPs (WHO, 2007). MDR-TB is a form of tuberculosis caused by strains of *M. tuberculosis* that are resistant to at least isoniazid (INH) and rifampicin (RMP) (Iseman, 1993). WHO has estimated that up to 50 million people may be infected with MDR-TB globally (WHO, 1998 b) and the SAARC region contributed 38% of it (Rijal *et al*., 2002). The surveillance report of the IUATLD/WHO, 2004 showed that 1.3% and 20% of untreated (new) and treated (old) cases had MDR-TB in Nepal.

Global Plan to Stop TB, 2006–2015 (Millennium Development Goal-MDG 6) has committed to achieve the following targets (WHO fact sheet, 2006):

- by 2005: detect at least 70% of new sputum smear-positive TB cases and cure at least 85% of these cases
- by 2015: reduce TB prevalence and death rates by 50% relative to 1990
- by 2050: eliminate TB as a public health problem (1 case per million population).

3.2 Historical Background

The disease tuberculosis has been a major cause of suffering and death since the time immemorial. It is though to be one of the oldest human diseases. There have been references to this ancient scourge in the Vedas (*Vide Infra*) and it was called “Rajayakshama” (meaning, “wasting disease”) (Sharma and Mohan, 2001). Hippocrates described the disease as “Pthisis”, a Greek word, which meant “to consume”, “to spit” and “to waste away” (Grange, 1996).

Changes resembling those caused by tuberculosis have been described in skull and other bone remains of Neolithic man. Evidences of tuberculosis lesions of bone have also been found in Egyptian mummies dating back to 3400 B.C (Keers, 1978). Terms such as “lung cough” and “lung fever” have been used in ancient Chinese literature to describe a disease, which may have been tuberculoses. *M. tuberculosis* has been

demonstrated microscopically in the mummies of a child about five years of age (Zimmerman, 1979).

The history of sputum examination dates back to March 24, 1882 when Robert Koch discovered the tubercle bacillus and confirmed the bacterial etiology of TB (Ponticeellio *et al*., 2001). The modern knowledge of tuberculosis started from the work of Rene Theodore Laennec, (1781-1826) a French clinician, who himself was consumptive and succumbed to the disease. In 1819, he invented the stethoscope which helps in accurate description of tuberculosis lesions; he described follicular (miliary) and infiltrative (exudative) forms of tuberculosis (Rao *et al*., 1981).

In 1876, Franciscus Sylvius, a Dutch physician, was the first to identify actual tubercles as a consistent and characteristics change in the lungs and other areas of consumptive patients. He also described that its progression led to abscesses and cavities (Lowell, 1966). J.L. Schonein, Professor of medicine at Zurich, was credited to have named the disease "tuberculosis" (Roseenblatt, 1973). The word "tuberculosis" means "a small lump" (Dubos and Dubos 1953; Waksman, 1964). The acid fast nature of the organism was discovered by Ehrlich in 1882 and the acid fast staining was developed by Ziehl (1882) and subsequently modified by Neelsen and hence the named Ziehl-Neelsen stain. X-ray was discovered in 1895 by Professor Roentgen and was put to clinical use by 1904. The findings of radiology and bacteriology helped in developing further knowledge of the disease and correlation between them (Rao *et al*., 1981).

3.3 Mycobacterium

The genus *Mycobacterium* is composed of slow growing acid fast organisms. It is the only member of the family Mycobactericeae of the order Actinomycetales (Ratledge, 1982); although there has been the proposal to include the genera *Nocardia* and *Rhodococcus* in this family. Currently about 71 species of mycobacteria are recognized (Good and Shinnick, 1998). The generic name of *Mycobacterium* which means 'fungus bacterium' was proposed by Lehmann and Neumann (1896) in reference to the mould like pellicle formed by M. *tuberculosis* in liquid media (Grange, 1990). Members of the genera *Mycobacterium* are very thin rods with rounded extremities, pleomorphic, straight or slightly curved (Ananthanarayan and Paniker, 1996; Forbes *et al*., 2002) gram-positive, non-motile, catalase positive and occasionally of filamentous morphology with the typical "acid-fast" staining characteristic. The optimal growing

temperatures of family Mycobactericeae vary widely according to different species, from 25°C to over 50°C under aerobic to microaerophilic condition (5-10% CO2), with generation times from 2 hours to more than 20 hours. Most of the members of mycobactericeae are saprophytes and adapt readily to growth conditions on very simples substrates, using ammonia or amino acids as nitrogen source and glycogen as carbon source. Only some of them are facultative pathogenic (Schliesser, 1985).

Among the *Mycobacterium* species, *M. tuberculosis* is weakly Gram positive, aerobic or microaerophilic, non-sporing, non-capsulated, straight and slightly curved rod (Good and Shinnick, 1998) measuring 1-4 x 0.2-0.5 µm (Cheesbrough, 2000). Tubercle bacilli grow slowly with generation time 12-24 hours, colonies usually appears in 2-3 weeks and may sometimes requires 8 weeks incubation, optimum temperature 37°C and pH 6.4-7.0 (Chakraborty, 2003). In sputum and other clinical specimens, they may occur singly or in small clumps, and in liquid cultures they often grow as twisted rope like colonies termed 'serpentine cords' (Greenwood *et al*., 2002). The colonies of *M. tuberculosis* on solid medium are dry, rough, and creamy or buff colored (Cheesbrough, 2000). The virulence of *M. tuberculosis* have been correlated with the formation of long cord like structure on agar or in liquid medium which is due to side to side aggregation and intertwining of long chains of bacteria. Growth in chords reflects the presence of a characteristics lipid, the cord factor, a glycolipid on the cell surface (Madigan *et al*., 2000). Non-acid fast rods and granules from young culture are also reported and when they are injected into susceptible animals, they produce tuberculosis. Perhaps these granules are non-acid fast form of tubercle bacilli. These are called Much's granules (Satish, 1999).

3.3.1 Cell wall structure

The mycobacterial cell wall structure has many similarities with that of other bacteria. The cell wall of *M. tuberculosis*, like that of other mycobacteria has a total thickness about 20 nm and appears to consists of an inner, electron dense layer surrounded by an outer, electron transparent layer (Brennan and Draper, 1994). The inner membrane or plasma membrane which limits the cytoplasm has the classic triple layered shape. The cell wall of mycobacteria contains a high amount of long chain peptidoglycolipid (up to 24% of 60-90 carbon lipids) termed mycolic acid (Wilson and Miles, 1964).

The basis of backbone structure of mycobacteria is the peptidoglycan. The cell wall of *M. tuberculosis* contains N-glcolymuramic acid instead of N-acetylmuramic acid (Jolik *et al.*, 1992). The lipopolysaccharide is made of arabinogalactan, a polysaccharide present only among bacteria of genera *Mycobacterium*, *Corynebacterium* and *Nocardia*, which is esterified at its distal end with mycolic acids. Mycobacteria contain a lipid rich cell surface which includes pure waxes and glycol-lipids, accounting for about 60% of the cell as dry weight (Barsdale and Kim, 1977). The long chain (60-90 carbon) mycolic acid in cell wall is composed of saturated α-alkyl, β-hydroxyl fatty acids. This lipid rich cell surface is responsible for acid fastness, failure to react with Gram stain and probably for resistance to the bactericidal action of antibody and complement (Madigan *et al*., 2000).

M. tuberculosis is also capable of synthesizing a wide variety of other complex molecules, associated with the outer layer of cell wall (Minnikin, 1982). Among them, sulfatides of sulfated acyltrehalose might play a role in virulence because they prevent phagosome fusion in cultured macrophages infected with *M. tuberculosis*. The "cord factor", a trehlose dimycolates, has been demonstrated in all mycobacterial species. Several waxes, especially phtiocerol, dimycocerosater and triacyl-glycerols or triglyceroles have been isolated from *M. tuberculosis* strains. Their presence increases the overall impermeability of the mycobacterial cell wall (Hal *et al*., 1987).

The high content of lipids on the cell wall is responsible not only for the extreme hydrophobicity of mycobacterial cells but also for the resistance of mycobacteria to chemical and physical injury; in other words, to decontamination procedures using sulfuric acids, sodium hydroxide, and detergents. On the other hand, it explains why *M. tuberculosis* is susceptible as other bacteria to heat and UV rays and alcohol. In acidic conditions, for example in 2.5-5% oxalic acid or 3-6% sulphuric acid, at least 30 minutes are needed to inactivate *M. avium* (Schliesser, 1985). Temperature of 60°C for 20 minutes and moist heat at 100°C kills it. In sun light the culture may be killed in 2 hours. In sputum it survives 20-30 hours even in sunlight. It is killed by tincture iodine in 5 minutes and 80% ethanol in 2 to 10 minutes. Phenol solution (5%) kills it in 24 hours. Mycobacteria can survive in refrigerator condition (4-10oC), in general, for more than 6 months without any loss of the ability to multiply. To inactivate mycobacteria by heat, 70°C and 65°C for 5 and 15 minutes, respectively, are needed (Schliesser, 1985).

3.3.2 Acid fast property

Acid fastness, the most prominent feature of the genus, is the ability to resist decolorization by weak mineral acid after being stained by an arylmethane dye. They resist decolorization because they strongly bind to the mycolic acid and cannot be removed during decolorization with 3% acid-alcohol solution (Salfinger and Kafader, 1992). The exact basis of acid fast staining reaction is not clearly understood, however it is related to the presence of mycolic acid in cell wall (Good and Shinnick, 1998). Several observations have led to a working model for the acid fast stain. First, mycobacteria with ruptured cell walls are weakly acid fast and treatment with alkaline ethanol renders them non acid fast. Secondly, free mycolic acids bind fuchsin on a mole basis; after mycolic acids have formed complexes with arylmethane dyes, the cell surfaces become extremely hydrophobic. It is thought, therefore, that the intense carbol fuchsin staining after decolorization with acid alcohol is produced by the portion of stain that has penetrated the interior of the *Mycobacterium* which is protected by the hydrophobic complex of the fuchsin and mycolic acids on the cell's exterior. *Corynebacterium, Nocardia* and *Rhodococcus* which may be acid fast after exposure to mineral acid are unable to protect their cells form acid alcohol decolorization (Barksdale and Kim, 1997).

3.3.3 Slow growth

Slow growth is a central characteristic of the genus. On the basis of growth property mycobacteria are divided in two groups, which grow in less than 7 days (rapid growers) and those which require more than 7 days, usually 2-3 weeks or more to grow (slow growers) (Good and Shinnick, 1998). This characteristics is not well understood, but is believed to be associated with a DNA depended RNA polymerase, initiating RNA synthesis and producing RNA in *M. tuberculosis* at a rate at least 10 times slower than in *Escherichia coli* (Hal and Ramakrishna, 1977). The enzymes in *M. tuberculosis* are also 1000 times more sensitive to rafampicin compared with the *E. coli* DNA depended RNA polymerase (Wayne, 1982). *M. tuberculosis* is a typical slow grower and visible colonies are produced from a dilute inoculum only after weeks of incubation. When growing on solid media, mycobacteria generally form tight, compact, often wrinkled colonies; the organisms piling up in a mass rather then spreading out over the surface of agar (Madigan *et al.*, 2000).

3.3.4 Nutritional requirements

The mycobacteria vary enormously in their metabolic activities, nutritional requirements and rate of growth. The basic nutritional requirements for most mycobacterium species are similar to those of other bacteria which include carbon, nitrogen, oxygen, phosphorous, sulphur, iron, magnesium and various trace elements (Ratledge, 1982; Wheeler and Ratledge, 1994). *M. tuberculosis* and some other species require special protein enriched media containing egg, asparagines, potatoes, serum and meat extracts (Frobes *et al*., 2002).

Carbon source: Various species of *Mycobacterium* can grow on wide range of carbon sources such as carbohydrates, organic acids, n-alkanes, acylesters and CO2. Glycerol is used as a carbon source in most culture media as it is the only carbon source that can be utilized by all species of *Mycobacterium* (Good and Shinnick, 1998). Almost all rapidly growing mycobacteria use glucose, mannose and trehalose; use of other sugars of alcoholic groups (e.g., inositol, dulcitol, mannitol and sorbitol) are variable (David *et al*., 1978). Organic acids are also used as carbon sources in some culture media. Pyruvic acid is used for the cultivation of *M. bovis*. Oleic acid stimulates mycobacerial growth although it is toxic in high concentrations (Good and Shinnick, 1998).

Nitrogen sources: For laboratory media, the preferred nitrogen source is asparagine or glutamine, although mycobacteria can obtain nitrogen from many inorganic sources, including ammonium nitrate and nitrite; and from organic sources like pyrimidine, amides, amines, amino acids and nucleosides. A few species may be able to assimilate nitrate or nitrite through nitrate and nitrite reductases to generate NH3. Variation in nitrogen utilization has been widely used for identification purposes, commonly used tests include nitrate utilization of amino acids which varies between and within species (Good and Shinnick, 1998).

Iron: Iron is an essential requirement for bacterial growth and metabolism. Mycobacteria have evolved a sophisticated system for scavenging iron from the environment and their eukaryotic host. Mycobacteria secrete small water soluble iron binding sidophores called exochelins. The MS-type of exochelin (e.g. produced by *M. smegmatis*) remains water-soluble at all stages, whereas the MB-type (e.g. produced by *M. tuberculosis*) becomes chloroform extractable when complexed with ferric (Wheeler and Ratledge, 1994). Each type of exochelin actually encompasses a family of small

molecules whose masses range from approximately 0.7 to 0.8 kDa, which reflects varying numbers of CH2 groups on an alkyl side chain (Gobin and Moore, 1995). The mycobactins are water-insoluble lipids located on the cell wall. They are synthesized by all cultivable mycobacteria except *M. paratuberculosis* and some stains of *M. avium*, and thus growth of these bacilli requires the addition of mycobactins to culture medium. The mechanism of their acquisition by these strains is unknown (Good and Shinnick 1998). Iron is rendered soluble and chelated from the external environment by exochelin and then transferred to mycobactin for transport across the cell wall. There is evidence that iron complexed to the MS-type exochelin may be transported directly across the cell wall, with the mycobactin serving merely as an iron store (Gobin and Moore, 1995).

Mycobacteria require several other inorganic elements for growth such as sodium, potassium, phosphorous, sulphur and magnesium as well as variety of elements, including zinc and manganese. CO2 is essential for optimal growth of many species and is usually included in media as NaHCO3/Na2CO3 or as CO2 in the gas phase at the concentration of 5-10% (Ratledge, 1982).

3.3.5 Classification of mycobacteria

Mycobacteria are grouped into two groups, ‘rapidly growing’ and ‘slowly growing’, based on the time required for colonies to appear on solid media. The slowly growing species are further divided into 3 groups based on pigmentation (Good and Shinnick 1998). According to the Runyon classification (Levinson and Jawetz, 1992), mycobacteria are classified as:

Runyon Group I: Those forming yellow to red pigment when cultured in the light, a property called photochromogenes (photo = light; chromogen = color producing). The group includes *M. kansasii*, *M. marium*.

Runyon Group II: Those forming reddish pigment in the light and yellow to orange pigment even when cultured in the dark, a property called scotochromogenes (scoto = dark; chromogen = color producing). The group includes *M. gordonae*, *M. parafiricum*.

Runyon Group III: Nonphotochromogens, which are weakly or non-pigmented even when cultured in dark or in light. The group includes *M. tuberculosis*, *M. bovis*.

Runyon Group IV: Rapid growers, in which growth is visible within a few days. The group includes *M. fortuitum, M. chelonae*.

3.3.6 Mycobacterium tuberculosis complex (MTC)

The *Mycobacterium* of clinical interest is divided into two groups; those associated with tuberculosis are called *M. tuberculosis* complex and other mycobacteria that may be associated with other human disease are called *Mycobacterium* other than tuberculosis bacilli (MOTT). Many MOTTs are found in the environment but they can also colonize in man; such as in the part of a previously damaged respiratory tract and cause clinical infection (Watt *et al*., 1996).

The organisms of the *M. tuberculosis* complex are always pathogenic for man. These include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*. These four species *of M. tuberculosis* complex are virtually identical in terms of DNA homology characteristics and antimicrobial susceptibility (Gross and Wayne, 1970; Wayne, 1982). But the organisms of the *M. tuberculosis* can be separated by several phenotypic and epidemiologic characteristics. Most human tuberculosis is caused by *M. tuberculosis*, but some cases are due to the *M. bovis*, which is principal cause of tuberculosis in the cattle and many other mammals. *M. bovis* rarely cause disease in areas of the world where animal husbandry includes TB screening and milk pasteurization (Des Prez and Heim, 1991). *M. microti* is believed to represent a transitional organism between *M. bovis* and *M. tuberculosis* but is not known to cause diseases in human. It is a pathogen of voles and other small mammals. *M. africanum* also causes human tuberculosis and mainly found in Equatorial Africa. It is intermediate in form between human and bovine type. Most of the *M. africanum* stains were similar to either *M. tuberculosis* or *M. bovis* (Braunstein *et al.*, 1990; David and Jahan *et al.*, 1978).

3.4 Diagnosis

The diagnosis of tuberculosis involves the detection and isolation of mycobacteria; identification of the mycobacterial species or complex isolated; and the determination of susceptibilities of the organisms to antimycobacterial drugs (ATS-CDC, 2000). The timely identification of a person infected with *M. tuberculosis* by rapid laboratory conformation and their treatment by appropriate chemotherapy are the two key ingredients of effective public health measure for the control of the TB (Noordeen and Godal, 1988).

Diagnostic testing for both latent tuberculosis infection (LTBI) and active disease has changed little during last century. LTBI, until very recently, have 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, NAA (Foulds and O'Brien, 1998).

3.4.1 Clinical Diagnosis

Clinical signs and symptoms develop in only a small proportion (5-10 %) of infected people (McMurray, 2001). Clinically, pulmonary tuberculosis is chiefly present with persistent cough for 3 or more weeks, shortness of breath and chest pain, loss of appetite and loss of weight, malaise, fatigue, night sweats and fever (Enarson *et al.*, 2000; WHO, 1997). The conventional methods includes tuberculin test, chest X-rays.

The tuberculin test relies upon procedures that use crude preparation of antigens to detect the cell-mediated immune response to the infecting *Mycobacterium*: the tuberculin, or purified protein derivative (PPD), skin test (ATS-CDC, 1981). However, the usefulness of tuberculin is limited by its lack of specificity for tuberculosis and its inability to distinguish between active disease and prior sensitization by contact with *M. tuberculosis*, BCG vaccination, and cross-sensitization by other *Mycobacterium* species (Huebner *et al.*, 1993). Chest X-rays help in the detection of pulmonary tuberculosis but they do not allow etiological diagnosis; there is the radiological difference between primary and secondary tuberculosis (McMurray, 2001).

3.4.2 Lab diagnosis

3.4.2.1 Specimens

The critical factor in the ability of a laboratory to isolate *M. tuberculosis* is obtaining an appropriate specimen for AFB smears and culture (Kent and Kubica, 1985). Because mycobacterial disease may occur in almost any site in the body, a variety of clinical materials may be submitted to the laboratory for examination. 85% of TB cases are pulmonary and most usual specimens for diagnosis of pulmonary tuberculosis is sputum If sputum is not produced, bronchial washing, brushing, transbronchial biopsy and other biopsies, bronchoalveolar lavage, laryngeal swabs and early morning gastric aspirated may also be examined (Bass *et al*., 1990). The remaining 15% of cases involve other sites and specimens which include urine, cerebrospinal fluid, pleural fluid, bronchial

washings, material from abscesses, endometrial scrapings, bone marrow, blood clots and other biopsy specimens or resected tissues. Tissue biopsies are homogenized by grinding for microscopy and cultures, cerebrospinal fluid, pleural fluid, urine and other fluids are centrifuged and the deposits are examined (Greenwood *et al.*, 2002).

Sputum is a thick fluid produced in the lungs and in the airways leading to the lungs. A good specimen of sputum should be mucoplurent, thick, yellow and sticky. It is the first choice in investigation of pulmonary disease and should be collected whenever possible, preferably before commencement of chemotherapy (Collins *et al.*, 1997).

It is best to collect the first specimen at clinic. The sputum, coughed up from deep in lungs is required, not saliva. Specimens should not be pooled; this could dilute a positive sample, increase contaminants and also introduce problems in preparing cultures. A wide mouth plastic container is generally used. The patient should be instructed to place the container against the lower lip and to expectorate carefully so that outside of the container is not contaminated. A good specimen for laboratory examination should be between 2 and 5ml (Collins *et al.*, 1997).

At least three consecutive daily sputum specimens may be necessary for diagnosis, as tubercle bacilli are released intermittently from lesions in the lungs. The examination of three specimens increases the predictive value of positivity of smear microscopy, reaching almost to that of culture (Chonde *et al.*, 2000). Three specimens have a 95% chance of recovery of tubercle bacilli. Whenever possible, they should be obtained within twenty-four hours (Akhtar *et al.*, 2000; WHO, 2000). The overnight specimen is more likely to be positive than the spot specimens. The cumulative positivity is 81%, 93% and 100% for first, second and third sputum, respectively (NTP, 2002). AFB microscopy can identify approximately 70% of culture positive cases of pulmonary tuberculosis. However, in practice, especially where direct smear are examined, the sensitivity falls well below that value (Foulds and O'Brien, 1998).

3.4.2.2 Microscopy

The detection of acid-fast bacilli (AFB) in stained smears examined microscopically is the first bacteriologic evidence of the presence of mycobacteria in a clinical specimen. It is the fastest, easiest, quickest and least expensive tool for the rapid identification of potentially infectious TB patients (Murray *et al*., 2003), and it provides the physician

with a preliminary confirmation of the diagnosis. Also, because it gives a quantitative estimation of the number of bacilli being excreted, the smear is of vital clinical and epidemiologic importance in assessing the patient's infectiousness (ATS-CDC, 2000). The main value of AFB microscopy lies in its speed and extremely high specificity, while the main disadvantage is said to be its low sensitivity. Sensitivity of microscopy is increased from 51% to almost 100% by introducing the cytocentrifugation (Kox, 1996).

Two procedures are commonly used for acid-fast staining: the carbolfuchsin methods, which include the Ziehl-Neelsen (ZN), also called "hot stain" and its modification, Kinyoun methods, also called "cold stain"(Cheesbrough, 2000); and a fluorochrome procedure using auramine-O or auramine-rhodamine dyes (ATS-CDC, 2000).

The "hot stain" requires application of heat to the fixed smears flushed with the stains during staining process. The Kinyoun stain is a modification of classical ZN method that excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin and phenol or the addition of detergent (Somoskovi *et al*., 2001). Recent advances in staining techniques have been reported where the cold Kinyoun stain has been further modified to accommodate the decolorizer within the counter stain and is called Modified cold stain. It is a two step cold staining method which requires concentrated carbol fuchsin as a primary stain and Gabbet methylene blue as counter stain (Bhat and Bhat, 2000).

Fluorescence microscopy was introduced by Hagemann (1937) who originally used berberine sulphate as the dye but later (1938) recommended auramine. Tryant *et al*. (1962) used two arylmethane dyes, auramine O and auramine B together. This combined staining could detect acid fast bacilli 358 of 3000 samples of sputum as against only 274 by Ziehl Neelsen method. In fluorochrome staining, smear is flooded with auramine, rinse with water and decolourize with 3% acid alcohol and again rinsed with water and then counterstained. The acid fast organisms appear as fluorescent rod, yellow to orange (the color may vary with filter system used) against a pale green background (ATS-CDC, 2000; Collins *et al.*, 1984; WHO, 1998a).

All of the above techniques are based on the relatively unique property of Mycobacterium to retain the primary stain even after exposure to strong mineral acid or

acid-alcohol (Sonnenwirth and Jarett, 1990). However, it is not definitive in terms of species identification and differentiation diagnosis (Woods and Washington, 1987).

3.4.2.3 Digestion and Decontamination of Specimens

The majority of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by more rapidly growing upper respiratory tract normal flora organisms (Karin *et al*., 2006a). Unless an attempt is made to inhibit these fast-growing contaminants, they can quickly overgrow the more slowly reproducing mycobacteria on the culture medium (ATS-CDC, 2000). The generation time for *M. tuberculosis* is18 to 24 hours, while it is only 40 to 60 minutes for other contaminating bacteria. This disproportionate rate of grow between the two types of microorganisms results in the accumulations of metabolic wastes products from the rapidly growing bacteria and thereby make the culture medium unsatisfactory for the growth of mycobacteria (Henry, 1991); they would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow. Most specimens must therefore be subjected to a harsh digestion and decontamination procedure that liquefies the organic debris (tissue, serum, and other proteinaceous material) surrounding the organisms in the specimen, so that the decontaminating agents may eliminate undesirable microbes and surviving mycobacteria may gain access to the nutrients of the medium onto which they are subsequently inoculated (Karin *et al*., 2006a). Since mycobacteria are more refractory to harsh chemicals than most other microorganisms, chemical decontamination procedures have to be carefully applied to ensure the recovery of maximum acid-fast bacteria from clinical materials (ATS-CDC, 2000). As a general rule, a contamination rate of 2-3% is acceptable in laboratories that receive freshly obtained specimens. If, on the other hand, specimens (especially sputum) take several days to reach the laboratory or cannot be processed immediately, then losses due to contamination may be as high as 5%-10%. It is also important to note that a laboratory which experiences no contamination is probably using a method that kills too many of the tubercle bacilli (Collins *et al.*, 1997; Karin *et al*., 2006a).

Many different methods of homogenization and decontamination of sputum specimens for culturing have been described, but there is no universally recognized best technique. The choice of a suitable method is, to a large extent, determined by the technical capability and the availability of staff in a laboratory, as well as the quality and type of equipment available (Karin *et al*., 2006a).

On arrival in the laboratory, most specimens are generally homogenized with a mucolytic agent (such as N-acetyl-L-cysteine) and decontaminant (such as a 1-2% sodium hydroxide solution) to render the bacterial contaminants nonviable. However, even under optimal conditions, these procedures kill all but 10 to 20% of the mycobacteria in the specimen (Roberts *et al*., 1991). Methods used in this study are Nekal-BX method and Petroff's (4% NaOH) method.

Petroff's method is the most popular method, devised in 1915 by Petroff and is suitable for material that is likely to be heavily contaminated. In this method, equal volume of 4% NaOH solution is added to about 4 ml of sputum in an appropriate container and neutralized with 1 N hydrochloric acid (Hans *et al.*, 1998; Petroff, 1915). A modified version in which the tube is centrifuged at 3000g for 15 minutes and the sediment is directly inoculated in the culture media (WHO, 1998b). When Ogawa media is used as the culture media, the sputum is directly inoculated in culture media after treatment with 4% NaOH, without undergoing the centrifugation procedure. The last method, in which sputum is directly inoculated in culture media after treatment with 4% NaOH, is used in the present study.

Different types of detergents have been proposed for use in the pretreatment of clinical material for cultural demonstration of *M. tuberculosis*. One of such substances is the anionic detergent, Nekal BX (diisobutyl naphthaline sulphonate), which in combination with 0.5% sodium hydroxide and 0.1% Na3PO4, is capable of homogenization and decontamination of the material within 16-18 hours of action. At the same time, the mycobacteria are accumulated in the sediment by precipitation in the presence of barium, calcium, and phosphate ions present in the Nekal BX reagent. The 16-18 hours of action ensures the killing of most of the contaminating agents while the majority of the resistant mycobacteria survive resulting in the high amount of the culture positive result (Warlo and Doerr, 1976).

Besides Nekal BX and Petroff's method, others methods employed in lab are N-Aceyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method, trisodium phosphate method, Zephiran-Trisodium Thiosulphate (Z-TSP) method, oxalic acid method, sulphuric acid method, Cetylpyridinium Chloride-Sodium Chloride (CPC) method etc. (Collins *et al.*, 1997; Kent and Kubica, 1985).

3.4.2.4 Cultural techniques

In 1882 Robert Koch reported the *in vitro* isolation of *M. tuberculosis* on an enriched bacteriological media. Koch originally grew the tubercle bacillus on heat-coagulated bovine or sheep serum, a culture medium invented by the Irish physicist John Tyndall. Nocard later introduced glycerol-beef broth which Koch used for his studies on tuberculin. Subsequently two main classes of media were developed: those based on heat coagulated (inspissated) egg; and synthetic of semi-synthetic media used in the fluid form or solidified with agar. The modern mycobacteriology laboratory have sophisticated laboratory media available to it, but in many respects TB isolation is carried out in a similar way as was pioneered by Koch (Tenover *et al.*, 1993).

The definitive diagnosis of tuberculosis demands that *M. tuberculosis* be recovered on culture media and identified using differential *in vitro* tests (WHO, 1998b). The inoculation of concentrated bacilli from processed clinical specimens on solid media is a standard approach for confirmation of TB (Kar *et al*., 2003). All clinical specimens suspected of containing mycobacteria should be inoculated (after appropriate digestion and decontamination) onto suitable culture media. Sputum culture to isolate mycobacteria is a highly sensitive diagnostic method that permits detection of a minimum of 10 to 100 viable bacilli in the given volume of cultured. The sensitivity of culture is excellent, ranging from 80% to 93 % (ATS-CDC, 2000; Dalovisio *et al.*, 1996). Moreover, the specificity is quite high, at 98 %. This is distinct from smear microscopy, in which, to achieve a 50% probability of observing one or more bacilli per 100 fields (positive result) the specimen must have at least 5,000 AFB/ml (ATS-CDC, 2000).

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. *M. tuberculosis* requires special media not used for other organisms and grows slowly, taking three to six weeks or longer to give visible colonies. Cultures are usually made in tubes rather than in petri dishes because tubercle bacilli are present in relatively small numbers in most specimens; this necessitates large inoculum which is spread out over the surface of the media. Because of the long incubation time required, the tubes are tightly capped to prevent drying of the cultures, which would otherwise occur in petri dishes (Karin *et al*., 2006a).

Many different culture media have been devised for growing the tubercle bacillus and other mycobacteria but relatively few of them are in use today. Those currently used as the first choice are the egg-based media. The culture is essential for following reasons:

- culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria/ml of material (Yeager, 1967)
- growth of the organisms is necessary for precise species identification
- drug susceptibility testing requires culture of the organisms
- genotyping of cultured organisms may be useful to identify epidemiological links between patients or to detect laboratory cross-contamination. (Ichiyama, 1993; Morgan *et al*.,1983; WHO, 1998b)

Characteristics of an ideal Culture media

The ideal medium for isolation of tubercle bacilli should:

- be economical and simple to prepare from readily available ingredients
- inhibit the growth of contaminants
- support luxuriant growth of small numbers of bacilli
- permit preliminary differentiation of isolates on the basis of colony morphology

For the culture of sputum specimens, egg-based media are the first choice since they meet all these requirements. There are increasing evidences that liquid media give better results with other specimens. While cost prevents their routine use with sputum specimens, it is recommended that both egg-based and liquid medium be used for non-repeatable specimens such as cerebrospinal fluid and biopsy material (Karin *et al*., 2006b).

3.4.2.4.1 Types of culture media

Many different media have been devised for cultivating tubercle bacilli out of which two main groups can be identified; they are solid media and liquid media.

3.4.2.4.1.1 Solid media:

Solid media include egg based media like Lowenstein-Jensen (LJ) medium, Ogawa medium and agar based media like 7H10 and 7H11. The culture of mycobacteria takes longer in solid media than in liquid media. In fact, LJ, 7H10 and 7H11 media may detect mycobacteria in less than 4 weeks, but they require incubation for as long as 6 to 8 weeks before they can be classified as negative (Morgan *et al.,* 1983)

3.4.2.4.1.1.1 Egg based media:

The egg-based media are represented by Ogawa, LJ and the various modifications of LJ media. Whole egg homogenate (in LJ media) or the egg yolk (in Ogawa media) is used in egg based media. The egg provides fatty acid and protein required for the metabolism of mycobacteria and the coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes (Difco/BBL).

3.4.2.4.1.1.1.1 Lowenstein-Jensen Media:

Lowenstein-Jensen Medium is exclusively used for the cultivation of *M. tuberculosis* and other mycobacterial species. Lowenstein Medium Base is a relatively simple formulation that requires supplementation in order to support the growth of mycobacteria. Lowenstein originally formulated a medium for cultivation of mycobacteria; in which congo red and malachite green were incorporated for the partial inhibition of other bacteria. Malachite green selectively inhibits contaminants (Difco/BBL). A good LJ medium should be non-selective, light green in color, smooth slant without bubble formation so as to view mycobacterial growth easily. The concentration of malachite green is critical for achieving a good color contrast for visualization of mycobacterial colonies. Sub optimal concentration of malachite green in the medium produces higher contamination rates where as excessive malachite green can suppress and delay the *Mycobacterium* growth itself (Tulip Diagnostics).

3.4.2.4.1.1.1.1.1 Modification of LJ Media

Time and again the Lowenstein-Jensen medium has been modified in various ways by different researches to achieve different objectives and has been used as basis for selective, differential and enriched media for growth of different mycobacteria species.

The MLJ medium has been recommended by International Union against Tuberculosis and Lung Disease (IUATLD) to be used for the routine culture of *M. tuberculosis.* The IUATLD recommended MLJ medium consist of potassium dihydrogen phosphate anhydrous (2.4g), magnesium sulphate (0.24g), magnesium citrate (0.6g), asparagine (3.6g) and glycerol (12ml) dissolved in 600ml distilled water and autoclaved at 121ºC for 30 minutes, which forms the mineral salt solution. To this mineral salt solution, 20ml of 2% malachite green and 1000ml of homogenized whole egg is added (WHO, 1998b).

The LJ medium base enriched with glycerol improves the growth of *M. tuberculosis*, and inhibits *M. bovis*. Addition of sodium pyruvate to the LJ base facilates the growth of *M. bovis* (Watt *et al.*, 1996).

Hughes, Dixon and Cuthbert reported that the addition of pyruvic acid to egg-based media resulted in improved recovery of tubercle bacilli compared to recovery on egg-based media supplemented only with glycerol (Dixon and Cuthbert, 1967; Hughes, 1966).

Gruft found that the addition of ribonucleic acid (0.05 mg/ml) increases the percentage of tubercle bacilli recovered from clinical specimens, compared to recovery on the standard LJ medium (Gruft, 1963).

LJ medium Gruft, modified by adding low-level concentrations of penicillin (50.0 units/mL) and nalidixic acid (35.0 mg/mL) was used to inhibit gram-positive as well as some gram-negative bacterial contaminants (Gruft, 1971).

LJ medium with iron was used to determine iron uptake for differentiation and identification of mycobacteria. Wayne and Doubek differentiated rapidly-growing from slow-growing mycobacteria based on iron intake (Wayne and Doubek, 1968).

LJ medium with 5% sodium chloride is also used to characterize certain strains of mycobacteria. It is based on the ability of most rapid growers to grow on 5% NaCl. The inability of *M. chelonae* subsp. *Chelonae* to grow on 5% NaCl helps to differentiate it from other members of the *M. fortuitum* complex (e.g., *M. fortuitum* and *M. chelonae* subsp. *Abscessus*) (Kent and Kubica, 1985; Silcox *et al.*, 1981).

Lowenstein-Jensen Mycobctosel medium with cycloheximide (0.4mg/ml), lincomycin (0.002mg/ml) and nalidixic acid (0.035mg/ml) was used in an attempt to control excessive culture contamination. (Collins *et al.*, 1997; Petran and Vera, 1971).

Zaher and Marks (1977) indicated that addition of hydrochloric acid to a modification of LJ medium may be used to avoid neutralization and centrifugation of the sputum homogenate decontaminated by use of NaOH (Zaher and Marks, 1977).

3.4.2.4.1.1.1.2 Ogawa media

In the 1940s, Oka and Katakura devised an egg medium in which the basal solution contained only monopotassium phosphate and sodium glutamate (Tatsuji, 1960). Originally called 3% KH4PO4 medium by Ogawa, the Japan Committee on the Guide for Examination of Tubercle Bacilli, in 1950, changed it to 3% Ogawa medium (Ang *et al.*, 2001). MOG containing 2% monopotassium phosphate is also commercially available. Sputum specimen is directly inoculated onto the medium after treatment with 4% NaOH; unlike in LJ media which requires centrifugation of specimen before inoculation. The elimination of centrifugation is of vital importance in hospital laboratories that do not have the equipment. In addition, Ogawa medium is cheaper than LJ media because it is made without asparagine and easy to prepare (Ang *et al.*, 2001). Ogawa medium contains egg yolk instead of whole eggs (Grange, 1990). It has more salts and malachite green, the basic inhibitor for bacterial contamination during incubation period. Mycobacterial growth is higher in the Ogawa culture medium (95.5%) than in the LJ (92.5%) and contamination rate in Ogawa is notably lower (6.3%) than in the LJ (9.9%) (PCHRD, 2003).

Advantages and disadvantages of egg-based media

Advantages

- It is easy to prepare
- It is the least expensive of all media available and supports good growth of tubercle bacilli
- It may be stored in the refrigerator for several weeks provided it was made from fresh eggs and culture tube caps are tightly closed to minimize drying by evaporation

- Contamination during preparation is limited because it is inspissated after being placed in tubes. In addition, the malachite green added to the media suppresses the growth of nonmycobacterial organisms (Kent and Kubica, 1985).

Disadvantages

- It may take as long as eight weeks before cultures become positive, especially if specimens contain few bacilli or if decontamination procedures have been used overly harsh.
- When contamination does occur, it often involves the total surface of the medium and the culture is usually lost.
- Drug susceptibility tests are more difficult to perform on egg media because the concentrations of certain drugs must be adjusted to account for their loss by heating or by interaction with certain components of the egg, such as phospholipids (Kent and Kubica, 1985).

3.4.2.4.1.1.2 Agar based media:

The second type of media is agar-based media. The most commonly used are Middlebrook 7H10 and Middlebrook 7H11. They are complex media and are available as dehydrated powders. They require the addition of an oleic acid-albumin-dextrose-catalase (OADC) supplement. These media may are not as successful as egg based media for primary isolation but they are more convenient in areas where egg based media cannot be made or are too bulky to import. When Middlebrook 7H-10 or Middlebrook 7H-11 medium is used for isolation, cultures must be incubated in an atmosphere of 10% CO2. Exposure of Middlebrook 7H-10 or Middlebrook 7H-11 agars to either daylight or heat results in the release of formaldehyde in sufficient concentration to inhibit the growth of mycobacteria. (WHO, 1998b)

3.4.2.4.1.2 Liquid media

Broth-based culture systems typically use 7H-12 liquid medium (Woods, 2002). Broth media formulations include both manual and automated systems using radiometric or colorimetric methods for detection of mycobacteria. When combined with DNA probes for rapid species identification, they are capable of producing positive results in 2 weeks or less for the vast majority of sputum smear-positive specimens, and within 3

weeks for smear-negative specimens (Schluger, 2003). The oxidation of palmatic acid to release 14C-labelled CO2 forms the basis of most automated systems. 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).

3.4.2.5 Biochemical Properties

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

2) Niacin test: In niacin test, the niacin production by the *M. tuberculosis* is detected. A positive reaction is show by yellow color formation when the fluid extract of the growth culture is mixed with 4% aniline and 10% cyanogens bromide solution. It is used for definitive diagnosis of *M. tuberculisis* (Kent and Kubiac, 1985). INH test strip is also used for detecting niacin and its metabolite in the aqueous extract of organism of medium (Kilburn and Kubiac, 1968).

3) Combined Niacin-Nitrate test: The combined niacin-nitrate rest is performed at CDCs because it provides more intensely colored positive reactions in both the niacin and nitrate test. The nitrate reduction test may be more intensely colored in their combined procedure because of the electron donors present in egg medium (Kent and Kubica, 1985). In this case in the 4-week old culture on LJ media 2.5 ml buffered nitrate substrate is added, placed in water bath for 2 hours and the extract is used to perform nitrate reduction and niacin tests (Kent and Kubiac, 1985).

4) Growth on Medium Containing p-Nitrobenzoic Acid (PNB): In laboratories where facilities and reagents for niacin and nitrate testing are not available,

identification of tubercle bacilli may be done by a combination of one or more of the catalase tests together with growth at 25°C on LJ medium and growth on LJ medium containing 500µg/ml p-nitrobenzoic acid at 37°C. *M. tuberculosis* does not grow within three days at 37°C and does not grow at all at 25°C or on PNB medium. It also does not produce yellow or orange pigment in the dark or after exposure to light (WHO, 1998 b).

5) 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. The test is usually performed by suspending growth in a mixture of Tween 80 hydrogen peroxides at room temperature; the appearance of bubbles is interpreted as a positive reaction. In catalase test at 68°C, the growth is incubated in phosphate buffer (PH 7) at 68°C in a water bath for 20 minutes and chilled to room temperature before addition of Tween 80. *M. tuberculosis* produces less than 45mm bubbles at room temperature but is catalase negative at 68oC.

6) Oxidase test: Isoniazide-sensitive *M. tuberculosis* is peroxidase positive, while unclassified isoniazid resistant stains of *M. tuberculosis* are peroxidase negative.

7) Test for iron uptake: The appearance of reddish brown color on culture medium containing 2% ferric ammonium citrate, due to the formation of iron oxide is interpreted as a positive test. *M. tuberculosis* and other slow growing mycobacteria give negative test most rapid growers give a positive reaction.

8) Tween hydrolysis: The test is carried out by suspending the growth in a mixture of Tween 80 buffer neutral red at 37°C. The reaction is red at 4 hours, 5 days and 10 day. A pink color indicates hydrolysis of Tween 80. *M. tuberculosis* does not hydrolyze Tween 80. This test is particularly useful on differentiating *M. scrofulaceum* (negative), from *M. gordonae* and *M. flavesvens* (Positive) (Forbes *et al*., 2002; Kent and Kubica, 1985).

3.4.3 Other methods of diagnosis

3.4.3.1 The immunological methods

Mycobacterial antigen is detectable in clinical specimens by use of specific antibodies in agglutination technique and Enzyme Linked Immunosorbent Assay (ELISA) (Wadee *et al.,* 1990). 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 38 kDa antigen from *M. tuberculosis*. The 10 kDa, 16 kDa and 24 kDa proteins isolated from *M. tuberculosis* were also useful in ELISA (Kox, 1996). ELISPOT test 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).

3.4.3.3 Tuberculostearic Acid (TBSA) 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 result (Bloom, 1994).

3.4.3.4 Molecular Methods

In the field of infectious disease testing, molecular diagnostic methods have been developed to replace conventional diagnostic methods. Molecular testing can yield genetic information about the virulence and antibiotic resistance of a particular microorganism (Bloom, 1994). By providing reliable and definitive identification, these methods would help in the patient management while monitoring drugs therapy and prophylactic measures (Sritharan and Sritharan, 2000). Molecular methods relay on extraction, desired/targeted nucleic acid amplification and detection of conserved gene sequences of *M. tuberculosis*. These methods provide rapid detection, identification and characterization of the *M. tuberculosis* strains. Different target sequences have been used to confirm the diagnosis of TB (Niemann *et al.,* 2000). Several molecular procedures useful for diagnosis of mycobacterial diseases include strand displacement amplification (SDA), polymerase chain reaction (PCR) amplification, transcription-mediated amplification (TMA), reporter phase systems, oligonucleotide ligation

amplification and Q-beta replicase amplification. The first four of these amplification systems are the best developed of the system for mycobacteria (Bloom, 1994).

In addition to the widely used PCR based detections, several other amplification methods have been invented. Among them Loop Mediated Isothermal Amplification (LAMP) is a novel nucleic acid amplification method in which reagents reacts under isothermal condition with high specificity, efficiency and rapidity (Iwamoto *et al.*, 2003). It is characterized by the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using auto-cycling strand displacement reaction. Amplification and detection of gene can be completed in a single step by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature at about 65°C. Simple incubator such as a water bath or block heater is sufficient for the DNA amplification. It provides high amplification efficiency, with DNA being amplified 109-1010 times in 15-60 minutes. Because of its high specificity, the presence of amplified product can indicate the presence of target gene (Eiken, 2005).

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials

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

4.2 Methodology

4.2.1 Study Site

The study was conducted at German-Nepal Tuberculosis Project-National Reference Laboratory (GENETUP-NRL), Kalimati, Kathmandu, Nepal, from December 2005 to September 2006.

4.2.2 Study Population

The following two groups of samples were included for this study:

A) Sputum smear positive samples (n=220) collected from smear positive sputum samples from patients visiting GENETUP-NRL.

(B) Sputum smear negative samples (n=105) collected from smear negative sputum samples from patients visiting GENETUP-NRL.

These samples were obtained from a total of 177 patients visiting GENETUP-NRL for sputum testing with suspicion of pulmonary tuberculosis.

4.2.3 Sample Collection

All the 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. About 2-5 ml of mucoid or muco-purulent sputum specimen from each patient was collected and the container was labeled with Lab No. and the collection date, the specimen number, the patient's information etc. were registered.

4.2.4 Sample Processing

The collected sputum samples were processed for microscopy and cultured on the same day of collection.

4.2.4.1 Sputum Smear Microscopy

Sputum smear was prepared on a clean, new and unscratched slide with the respective lab number at one end. An appropriate portion of sample was transferred to the slide with the help of a broken end of a wooden stick. The sample was smeared on the slide over an area of approximately 2.0 x 1.0 cm making it thin enough to be able to read through it. Then the smear was allowed to air dry for about 15 minutes without heating. Thereafter, the smear was heat fixed by passing the slide through a flame three to four times with smear facing upper side and then allowed to cool before staining. These entire steps were performed inside a safety cabinet (WHO, 1998 a).

Among the several methods of determining acid-fast nature of mycobacteria, fluorochrome method was applied in this study. The numbered smear was placed on a staining rack and flooded with auramine-O. It was then allowed to stain for 15 minutes. Then the smear was rinsed with distilled water and covered with 20% sulphuric acid for 5 minutes. The smear was rinsed again with distilled water and counterstained with 1% methylene blue for 2 minutes. After washing with distilled water, the smear was allowed to air-dry and examined directly under the UV microscope. About 300 visual fields (V.F.) were examined before reporting as negative for fluorescence microscopy. The light source was switched on for at least 10 minutes before examination of commenced in order to obtain the optimal intensity of illumination. The microscopy results were interpreted on the basis of IUATLD standard (Appendix V).

4.2.4.2 Culture

Total of 6 tubes were taken for each sample. Out of which two tubes containing MLJ, two tubes containing MOG and two tubes containing BLJ were selected and labeled accordingly.

4.2.4.2.1 Decontamination procedure

Two different types of decontamination procedure were used in this study. Before the pretreatment procedure the sputum sample separated into two equal halves using a

sterile cotton swab and transferred into two different graduated centrifuge tube of 15ml capacity.

For Petroff's modified method, about same volumes of 4% NaOH was added to sputum specimen, vortexed it until specimen was completely liquefied and digested, and then left to stand for 15 minutes at room temperature with occasional shaking.

For Nekal BX method (Akthar *et al.*, 2000) Nekal BX solution was added to the sample to make it 1:4 volumes and was mixed and homogenized well using cotton swab. The tubes were left in room temperature to stand for over night. The next day, the tubes were centrifuged at 3000x g for 20 minutes. The supernatant liquid was discarded and the pellet along with little amount of liquid retained was vortexed until the pellet completely liquefied. The sediment thus obtained was used for culture.

4.2.4.2.2 Inoculation and incubation

For culture, 0.1 ml of pretreated sputum was inoculated into each culture tubes containing MLJ, MOG and BLJ. The inoculum was evenly spread over on the surface of the medium. The inoculated slants were placed in the incubator at 37ºC by keeping in slant position with the caps loosely for 2 days to allow excessive moisture to escape. The caps were closed tightly when the surface of the media dried and the tubes were incubated up to at least 9 weeks. The tubes were examined on 3rd day for rapid growers and weekly thereafter for slow growers. If any colonies were seen at any stage, acid-fastness of bacilli was determined by smear examination of the growth. Results were reported negative when no colonies appeared even after observing weekly for 9 weeks. Grading of primary culture is given in appendix V.

4.2.4.3 Identification

The colony morphology was observed for all positive culture tubes, which, for *Mycobacterium tuberculosis* should be buff-yellow colored, rough, bread crumbs or cauliflower like colonies. The direct microscopic examination of each positive culture was performed by Ziehl-Nelsen Stain. The microscopy results were interpreted on the basis of IUATLD standard (Appendix V).

One ml of distilled water and few pieces of glass beads were taken in test tubes. A loopfull of colony from each positive tube was transferred into these tubes and vortexed to make the colonies homogenized.

From these homogenized samples, smears were prepared on new, clean and sterile slides and fixed over a hot glass plate. The slides were flooded with 1% Ziehl's carbol fuchsin. Then the slides were heated gently from underneath until the vapour started to rise and left for 15 minutes without heating. Then the slides were rinsed with distilled water and covered with 20% sulphuric acid for 10 minutes for decolorization. The slides were rinsed again and counterstained with 1% methylene blue for 1 minute and washed with distilled water to remove the stain. The slides were dried and examined under microscope using an oil-immersion lens. The microscopy results were interpreted on the basis of guidelines given by IUATLD (Appendix V).

4.2.4.4 Biochemical tests

20% of randomly selected culture positive tubes were subjected to the biochemical tests: p-Nitrobenzoic Acid (PNB) test, catalase test, Nitrate reduction test, Niacin strip tests were performed (Appendix IV).

4.2.5 Interpretation

The results were interpreted on the basis of the growth and contamination rates on MOG, MLJ and BLJ.

4.2.6 Statistical analysis

Statistical significance was analyzed by using Chi-Square ($\chi 2$) test. The results were considered significant if the P-values with less than 0.05. The Chi-Square tests are shown in (Appendix VI).

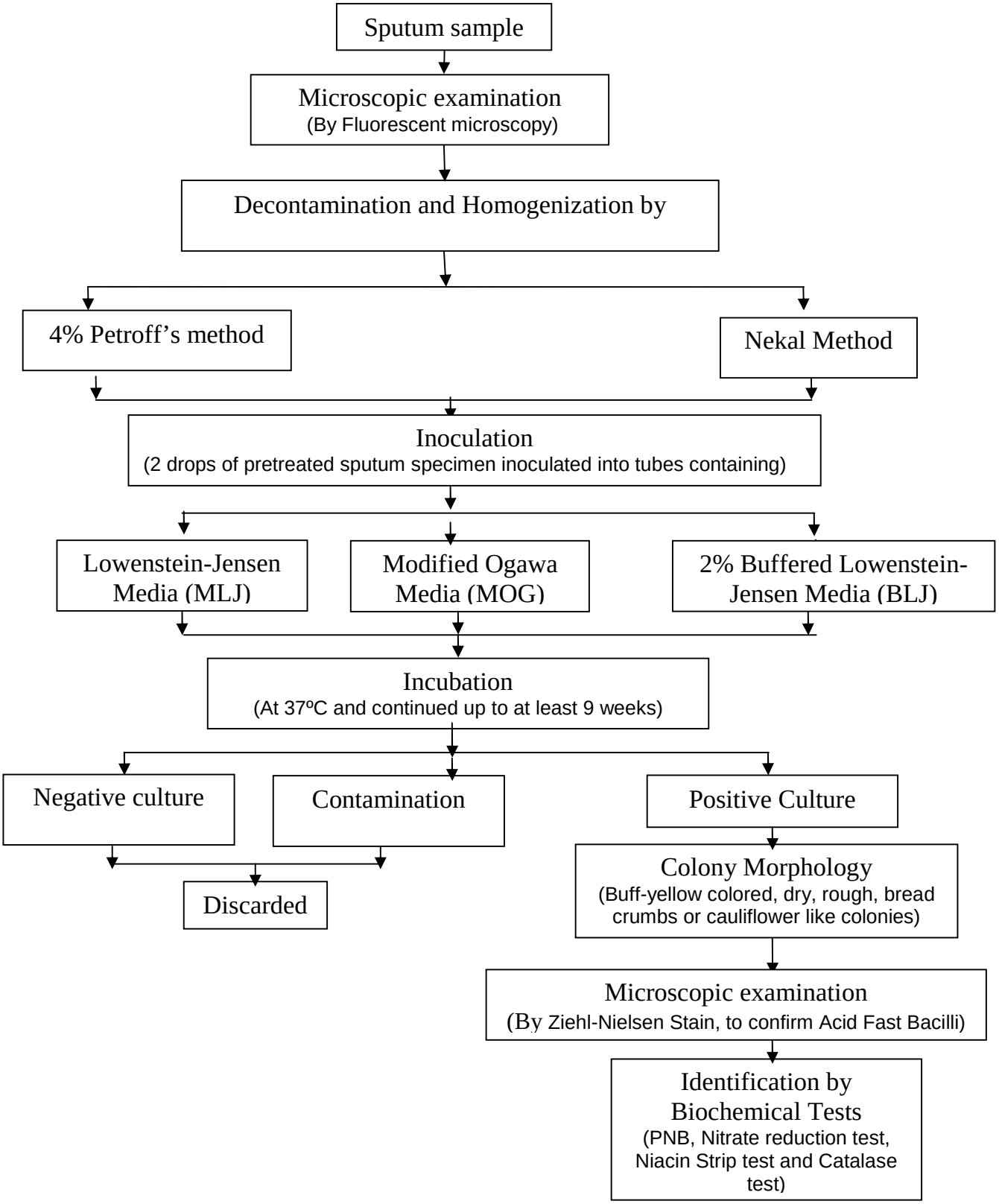


Figure 1: Schematic diagram of study design.

CHAPTER-V

5. RESULTS

In order to evaluate the efficiency of use of BLJ for the diagnosis of *M. tuberculosis*, a comparative study of BLJ against standard culture techniques of MOG and MLJ was performed. Simultaneously, the modified Petroff's (4% NaOH) method (without neutralization and centrifugation) was compared with the Nekal-BX method for homogenization of sputum. The sputum samples from the patients belonging to two different study groups were collected in this study. Out of 324 samples collected from 177 patients, 220 were from smear positive TB patients and 104 were from smear negative patients. All the samples were examined microscopically for AFB by standard fluorochrome method (WHO, 1998b) and cultured in MLJ, MOG and newly proposed BLJ media. The patterns of the growth and contamination rates were studied.

5.1 Laboratory Result of Samples

5.1.1 Study Group A

In this group, 220 smears positive samples were included, out of which 94.091% (207/220) were found to be positive by one or all culture, the remaining 5.91% (13/220) were negative. None of the samples was found to be contaminated in all six culture tubes.

Table 1: Culture positivity of smear positive samples from different media

	Positive (%)		Negative (%)		Contamination (%)	
Decontaminant / Media	4% NaOH	Nekal	4% NaOH	Nekal	4% NaOH	Nekal
MLJ	179 (81.3)	192 (87.27)	27 (12.27)	12 (5.54)	14 (6.36)	16 (7.27)
BLJ	203 (92.27)	204 (92.73)	10 (4.55)	9 (4.09)	7 (3.18)	7 (3.18)
MOG	201 (91.36)	204 (92.73)	14 (6.36)	10 (4.55)	5 (2.27)	6 (2.73)

Out of 220 (100%) smear positive sputum samples decontaminated using 4% NaOH, 91.36% (201/220) samples were positive on MOG whereas the 6.36% (14/220) showed no growth and 2.27% (5/220) samples were contaminated during the course of study. Similarly, 81.36% (179/220) samples were positive on MLJ, 12.27% (27/220) showed no growth and 6.36% (14/220) samples were contaminated. And 92.27% (203/220) samples were positive on BLJ, 4.55% (10/220) showed no growth and 3.18% (7/220) samples were contaminated.

Out of 220 (100%) smear positive sputum samples decontaminated using Nekal, 92.72% (204/220) samples were positive on MOG whereas 4.55%(10/220) showed no growth and 2.73% (6/220) samples were contaminated during the course of study. Similarly, 87.73% (192/220) samples were positive on MLJ, 5.45% (12/220) showed no growth and 7.27% (16/220) samples were contaminated. And 92.72% (204/220) samples were positive on BLJ, 4.09% (9/220) samples showed no growth and 3.18% (7/220) samples were contaminated.

5.1.1.1 Comparison of cultural Media

Out of 220 smear positive sputum samples 94.09% (207) were found to be positive in one or all culture media, while the remaining were negative.

5.1.1.1.1 Comparison between MLJ and MOG

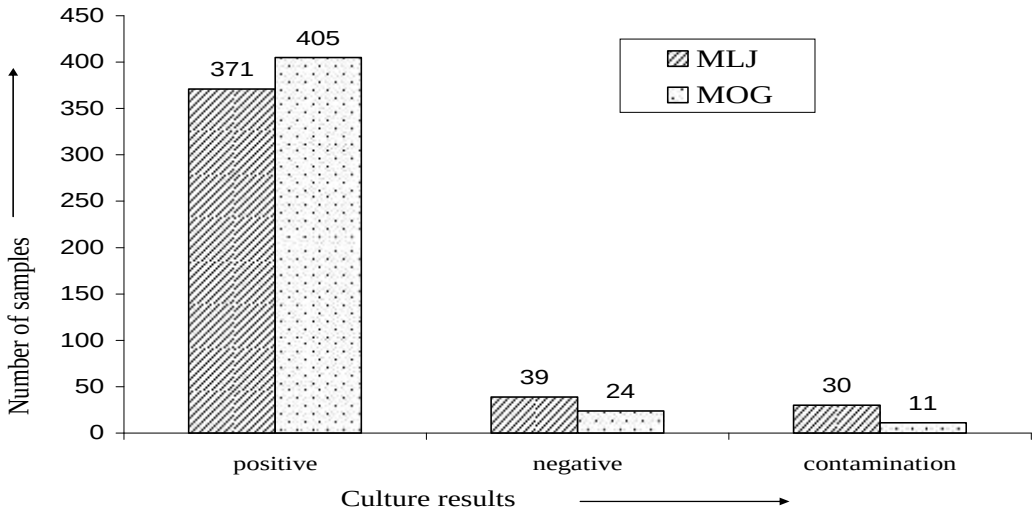


Figure 2: Comparison between MLJ and MOG.

When comparing the total results of 440 tubes of MLJ and MOG, 92.05% (405) samples were positive on MOG while only 84.32% (371) were positive on MLJ. Similarly, 5.45% (24) negative and 2.50% (11) were contaminated on MOG; and 8.86% (39) negative and 6.82% (30) contaminated on MLJ. The difference was significant at 99% level of significance (Figure 2).

5.1.1.1.2 Comparison between MLJ and BLJ:

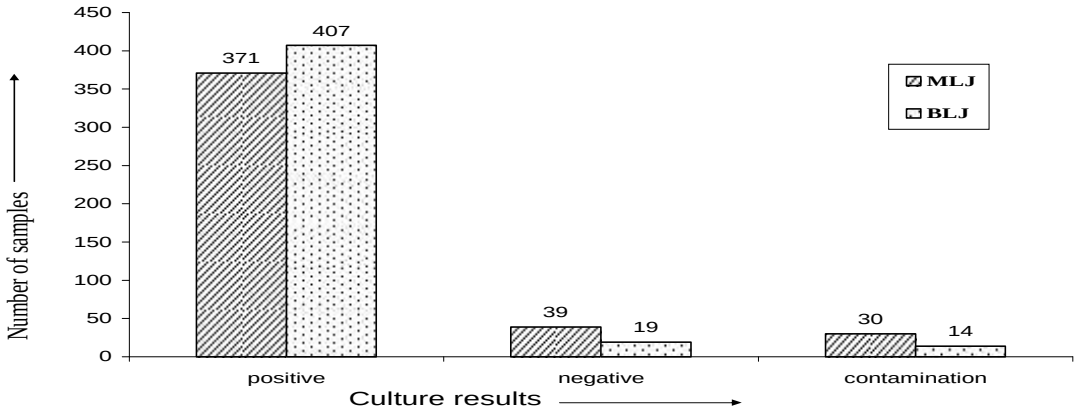


Figure 3: Comparison between MLJ and BLJ.

When comparing the total result of 440 tubes of MLJ and BLJ, 92.50% (407) samples were positive on BLJ while only 84.32% (371) were positive on MLJ. Similarly, 4.32% (19) negative and 3.18% (14) contaminated on BLJ; and 8.86% (39) negative and 6.82% (30) contaminated on MLJ. The difference was significant at 95% level of significance and also significant even at 99% level of significance (Figure 3).

5.1.1.1.3 Comparison between MOG and BLJ:

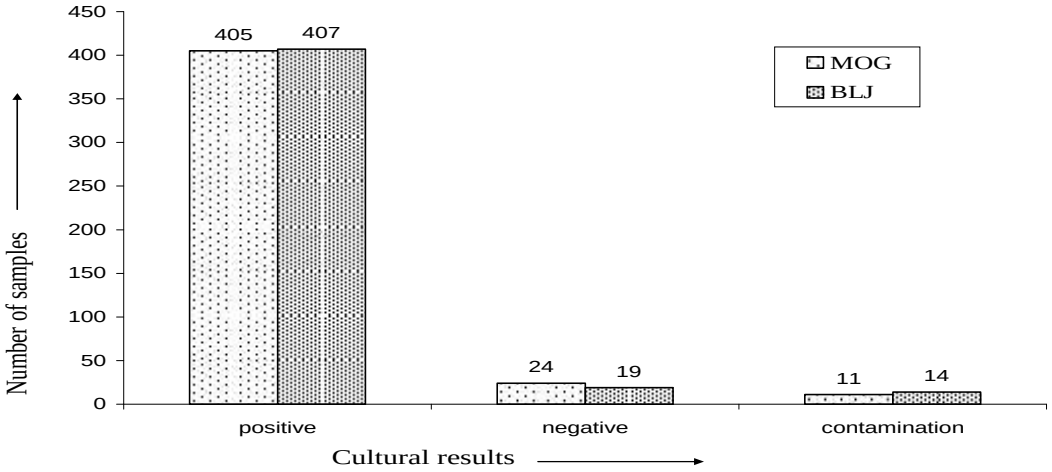


Figure 4: Bar diagram depicting comparison between MOG and BLJ.

When comparing the total result of 440 tubes of MOG and BLJ, 92.05% (408) samples were positive both MOG and BLJ. Similarly, 5.45% (24) negative and 2.50% (11) contaminated on MOG; and 4.32 % (19) negative and 3.18 % (14) contaminated on BLJ. The difference was not found to be significant (Figure 4).

5.1.1.2 Comparative recovery of bacteria in different culture media with decontamination by Nekal and 4%NaOH:

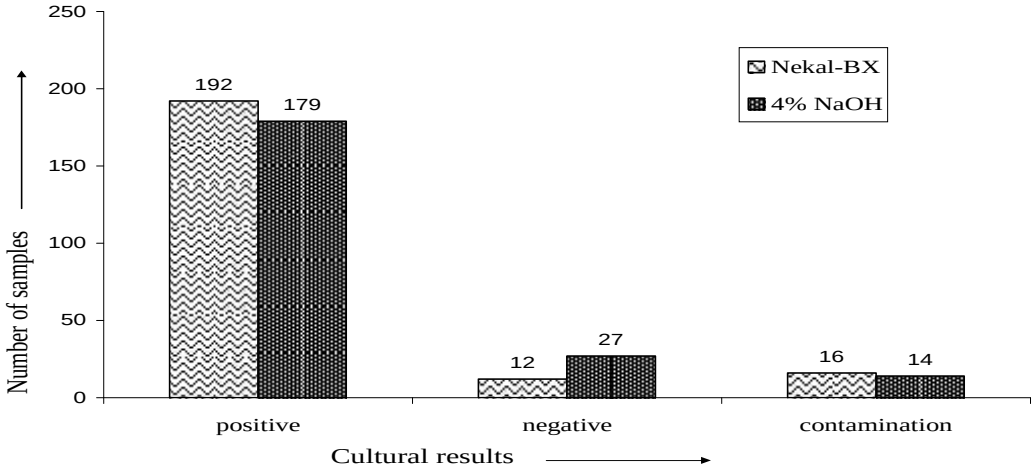


Figure 5: Comparison of decontaminating techniques on MLJ.

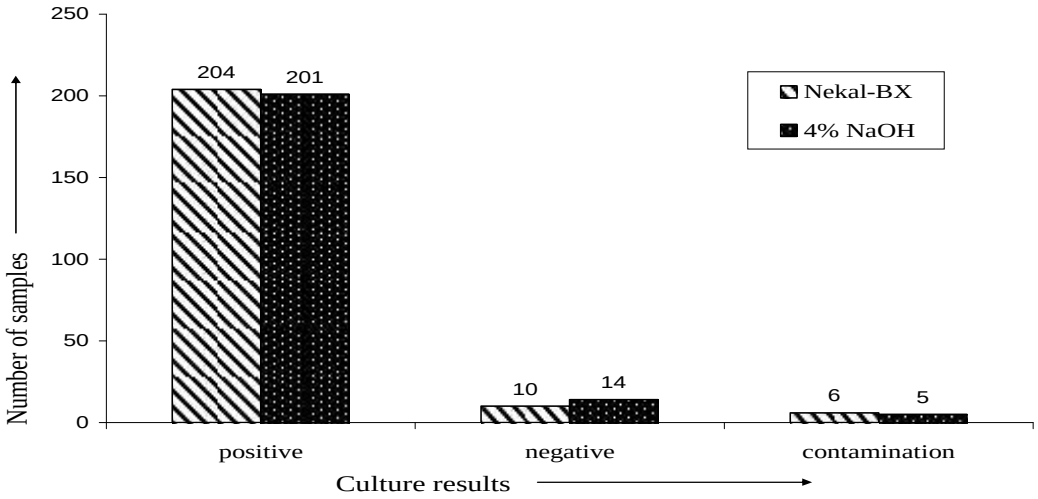


Figure 6: Comparison of decontaminating techniques on MOG.

When comparing the total result of 220 tubes of MLJ decontaminated by Nekal and 4% NaOH; Nekal gave 87.27% (192) positive results while 4% NaOH gave only 81.36% (179) positive results. Similarly, Nekal gave 5.45% (12) negative and 7.27% (16) contamination; and 12.27% (27) negative and 6.36% (14) contamination were given by 4% NaOH. The difference was significant at 95% level of significance (Figure 5).

When comparing the total result of 220 tubes of MOG decontaminated by Nekal and 4% NaOH; Nekal gave 92.73% (204) positive results while 4% NaOH gave 91.36% (201) positive results. Similarly, Nekal gave 4.55% (10) negative and 2.73% (6) contamination; and 6.36% (14) negative and 2.27% (5) contamination were given by 4% NaOH. The difference was not found to be significant (Figure 6).

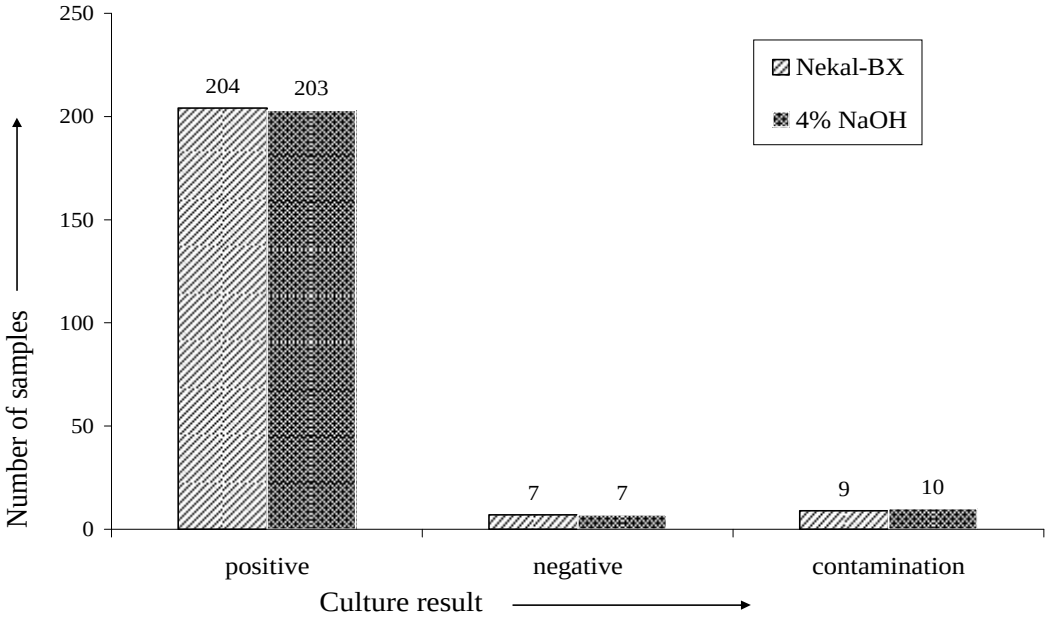


Figure 7: Comparison of decontaminating techniques on BLJ.

When comparing the total result of 220 tubes of BLJ decontaminated by Nekal and 4% NaOH; Nekal gave 92.73% (204) positive results while 4% NaOH gave 92.27% (203) positive results. Similarly, Nekal gave 3.18% (7) negative and 4.09% (9) contamination; and 3.18% (7) negative and 4.55% (10) contamination were given by 4% NaOH. The difference was not found to be significant (Figure 7).

5.1.1.3 Comparison of negative and Contamination rates:

For an ideal culture media, it is highly important that the media give as much less negative result and less contamination as possible to avoid the unnecessary repetition of culturing. The present study also intended to study the contamination rates and negative result among the three media compared.

5.1.1.3.1 Negativity rate:

The negativity rate was highest in MLJ with 4% NaOH as decontaminating agent (12.27%), followed by MLJ with Nekal as decontaminating agent (5.54%). When compared among BLJ and MOG the negative rate was higher in MOG (6.36% in 4% NaOH and 4.55% in Nekal) than in BLJ (4.55% in 4% NaOH and 4.09% in Nekal).

5.1.1.3.2 Contamination rate:

The contamination rate was highest in MLJ decontaminated by Nekal (7.27%) followed by MLJ decontaminated by 4% NaOH (6.36%). When compared among BLJ and MOG the contamination was higher in BLJ (3.18% in 4% NaOH and 3.18% in Nekal) than in MOG (2.27% in 4% NaOH and 2.73% in Nekal).

5.1.2 Study Group B

In this group, out of 104 smear negative samples, 9.62% (10/104) were found to be positive by one or all culture; the remaining 91.35% (95/104) were negative, and no sample was found to be contaminated in all six culture tubes.

Table 2: Culture positivity of smear negative samples from different media

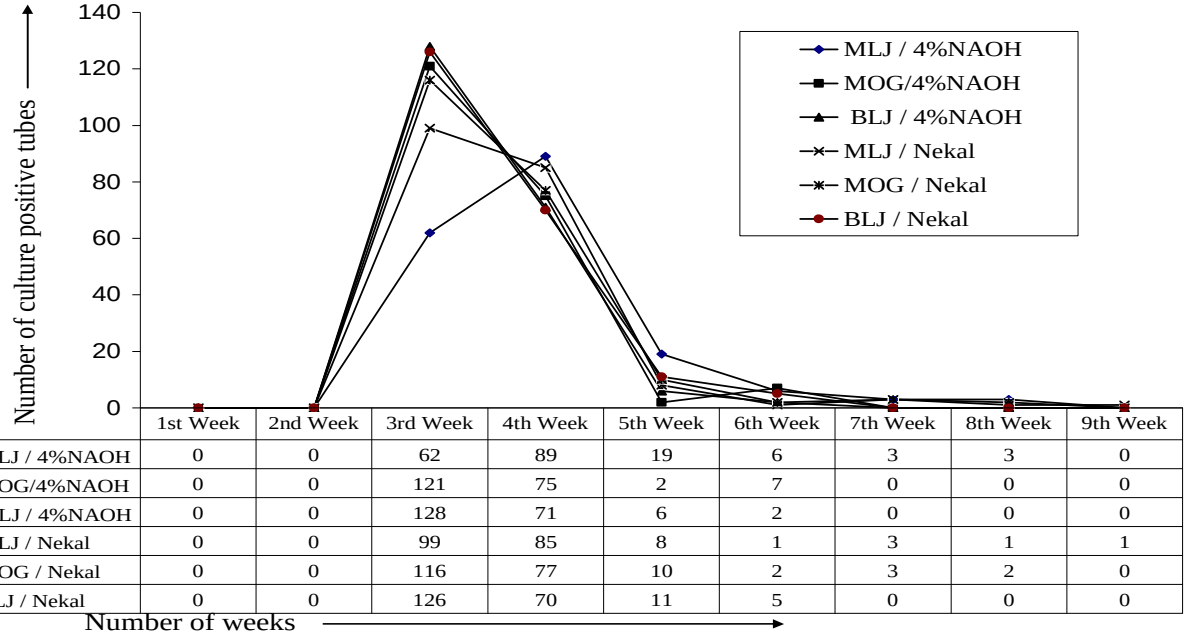
	Positive (%)		Negative (%)		Contamination (%)	
Decontaminant / Culture Media	4% NaOH	Nekal	4% NaOH	Nekal	4% NaOH	Nekal
MLJ	3 (2.88)	6 (5.77)	97 (93.27)	92 (88.46)	4 (3.85)	6 (5.77)
BLJ	4 (3.85)	6 (5.77)	93 (89.42)	90 (86.54)	7 (6.73)	8 (7.69)
MOG	4 (3.85)	5 (4.81)	95 (91.35)	93 (89.42)	5 (4.81)	6 (5.77)

Out of 104 (100%) smear negative sputum samples decontaminated using 4% NaOH, 3.85% (4/104) samples were positive on MOG 91.35% (95/104) of the samples were negative and 4.81% (5/104) samples were contaminated during the course of study. Similarly, 2.88% (3/104) samples were positive on MLJ 93.27% (97/104) of the samples were negative and 3.85% (4/104) samples were contaminated. And, 3.85% (4/104) samples were positive on BLJ 89.42% (93/104) of the samples were negative and 6.73% (7/104) samples were contaminated.

Out of 104 (100%) smear negative sputum samples decontaminated using Nekal, 4.81% (5/104) samples were positive on MOG 89.42% (93/104) of the samples were negative and 5.77% (6/104) samples were contaminated during the course of study. Similarly, 5.77% (6/104) samples were positive on MLJ 88.46% (92/104) of the samples were

negative and 5.77% (6/104) samples were contaminated. And, 4.81% (5/104) samples were positive on BLJ 86.54% (90/104) of the samples were negative and 7.69% (8/104) samples were contaminated.

5.1.3 Time of appearance of growth on culture positive tubes:



	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week	9th Week
MLJ / 4%NAOH	0	0	62	89	19	6	3	3	0
MOG/4%NAOH	0	0	121	75	2	7	0	0	0
BLJ / 4%NAOH	0	0	128	71	6	2	0	0	0
MLJ / Nekal	0	0	99	85	8	1	3	1	1
MOG / Nekal	0	0	116	77	10	2	3	2	0
BLJ / Nekal	0	0	126	70	11	5	0	0	0

Figure 8: Time of appearance of growth on culture positive tubes.

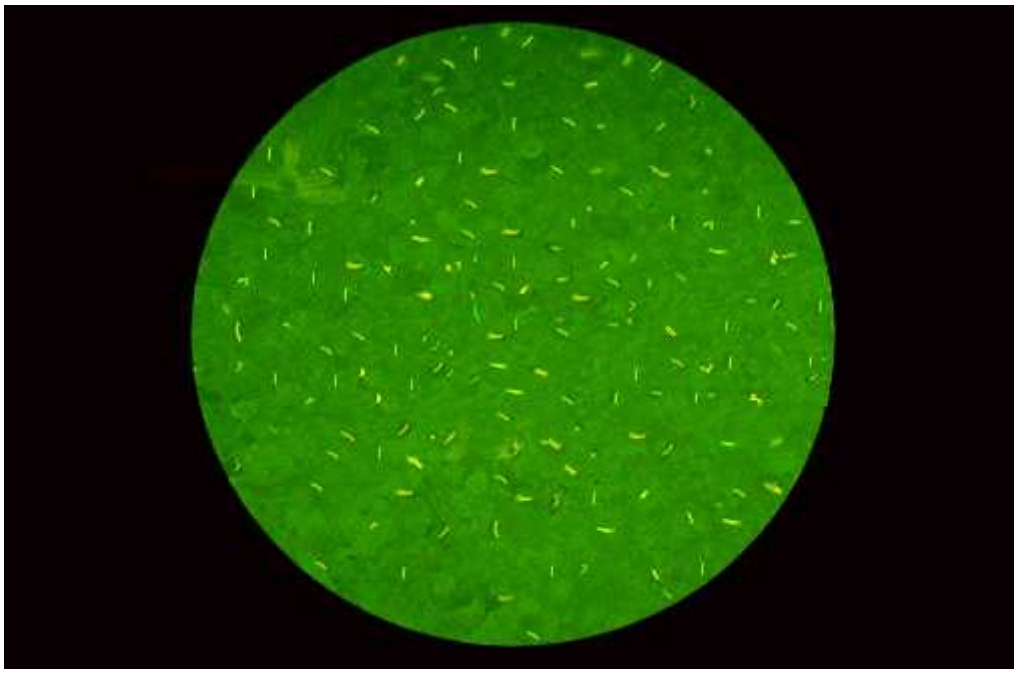
Out of total culture positive tubes the maximum growth was observed on 3rd, 4th and 5th week. No growth was observed before 3rd week.

5.2 Identification of culture:

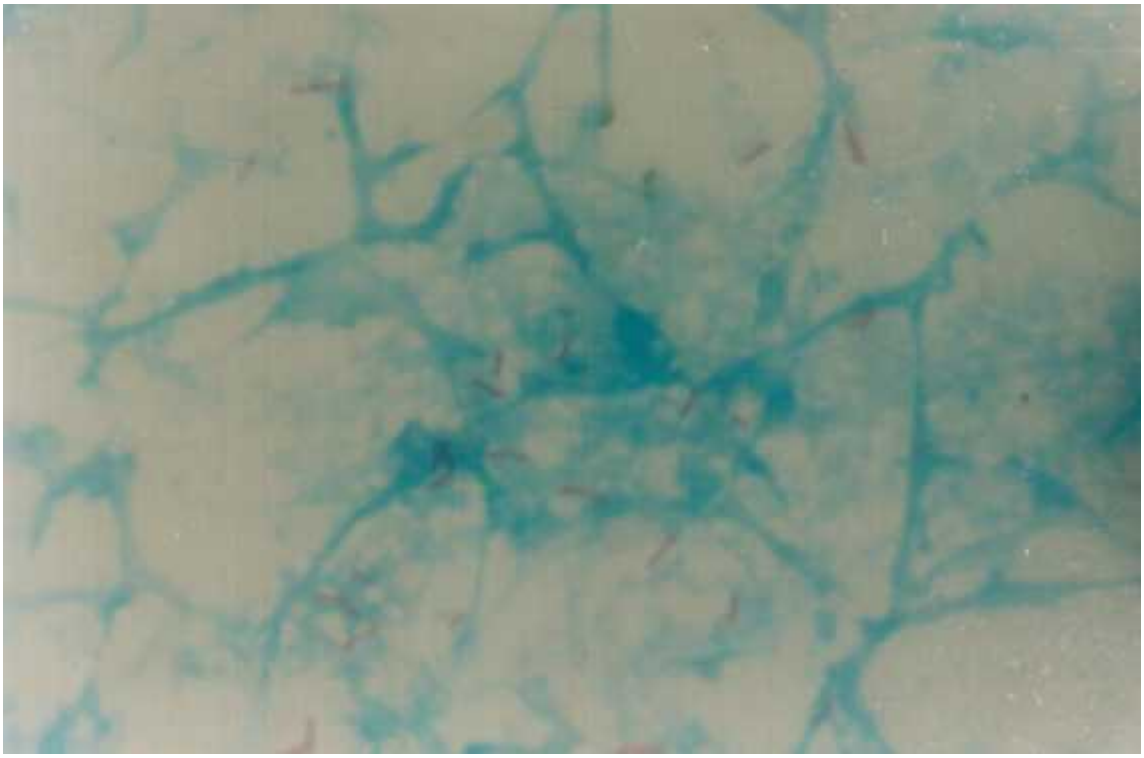
The cultures were identified as *Mycobacterium tuberculosis* on the basis of the colony morphology, Ziehl-Nelsen Staining and 20% (44 tubes) of all of the culture positives tubes were subjected to biochemical tests: p-Nitrobenzoic Acid (PNB) test, catalase test, Nitrate reduction test, Niacin strip tests. The colony morphology of the cultures were buff-yellow colored, rough, bread crumbs or cauliflower like colonies. The Ziehl-Nelsen Staining of the cultures showed red acid-fast organisms against the blue background of methylene blue. The *M. tuberculosis* bacteria were identified having typical serpentine coils. The sample tested positive for Niacin test and Nitrate reduction test, negative for heat labile catalase test and no growth on PNB containing medium.

Photograph 1: Investigator observing acid fast bacilli under fluorescent microscope

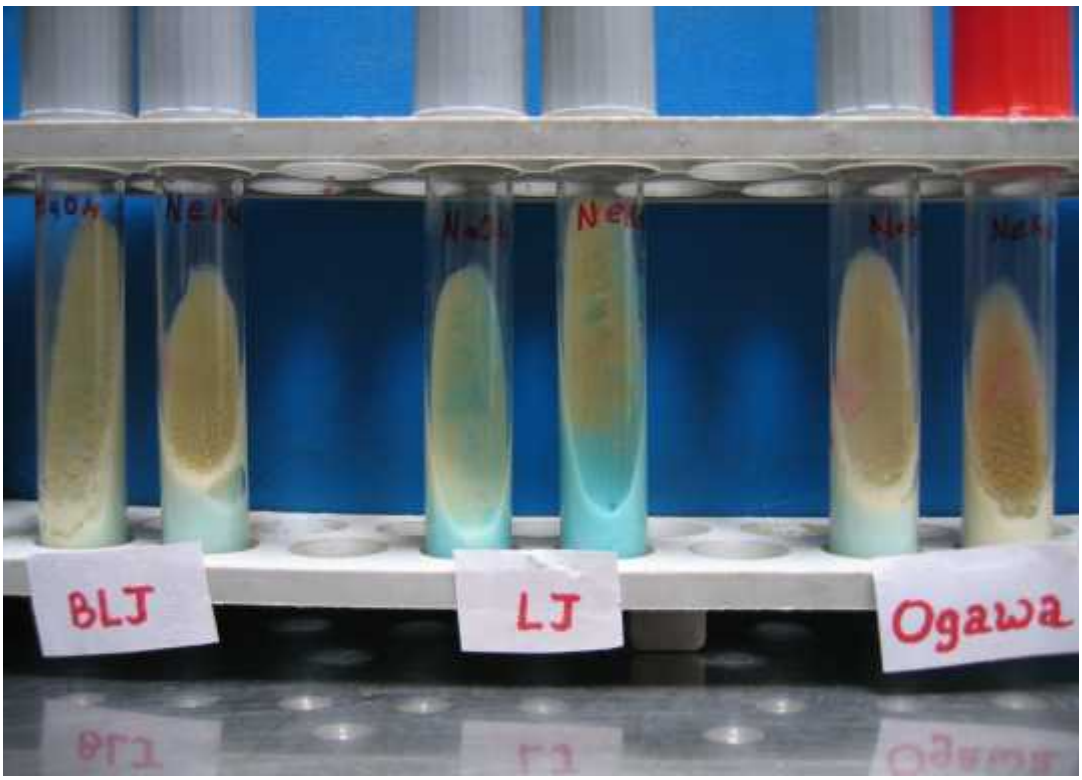
Photograph 2: Investigator working in Safety cabinet



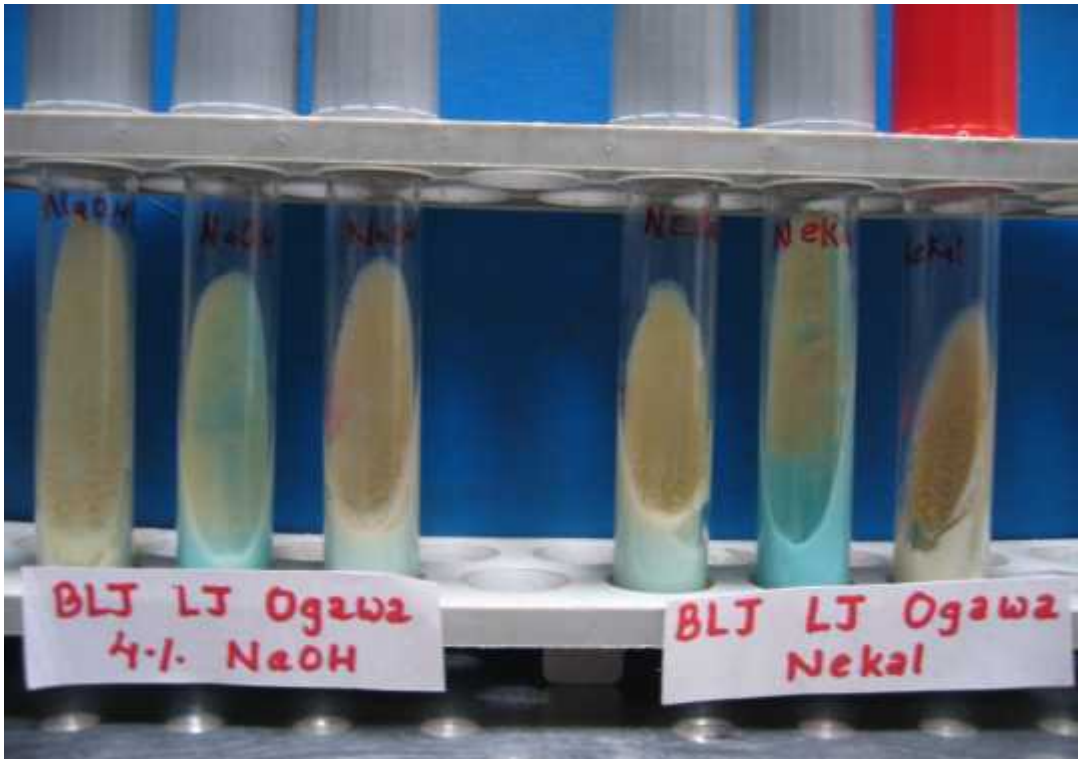
Photograph 3: AFB stained by Auramine staining



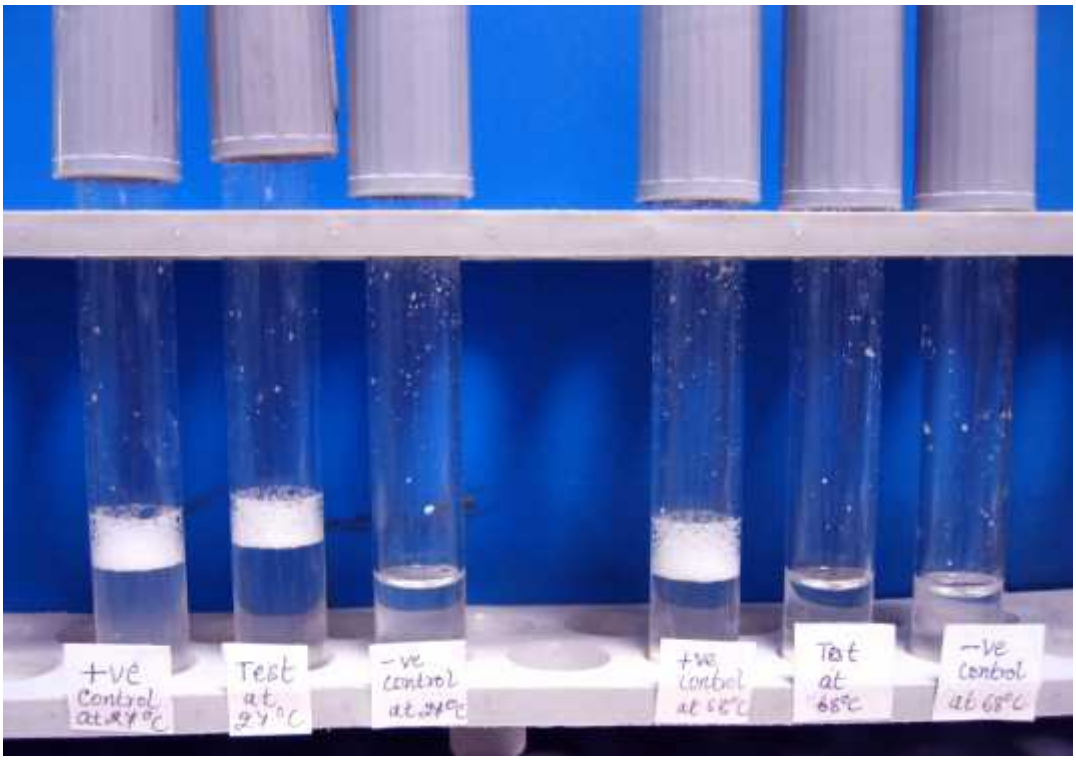
Photograph 4: AFB stained by Ziehl-Nelsen staining



Photograph 5: *M. tuberculosis* growth on culture tubes according to culture media

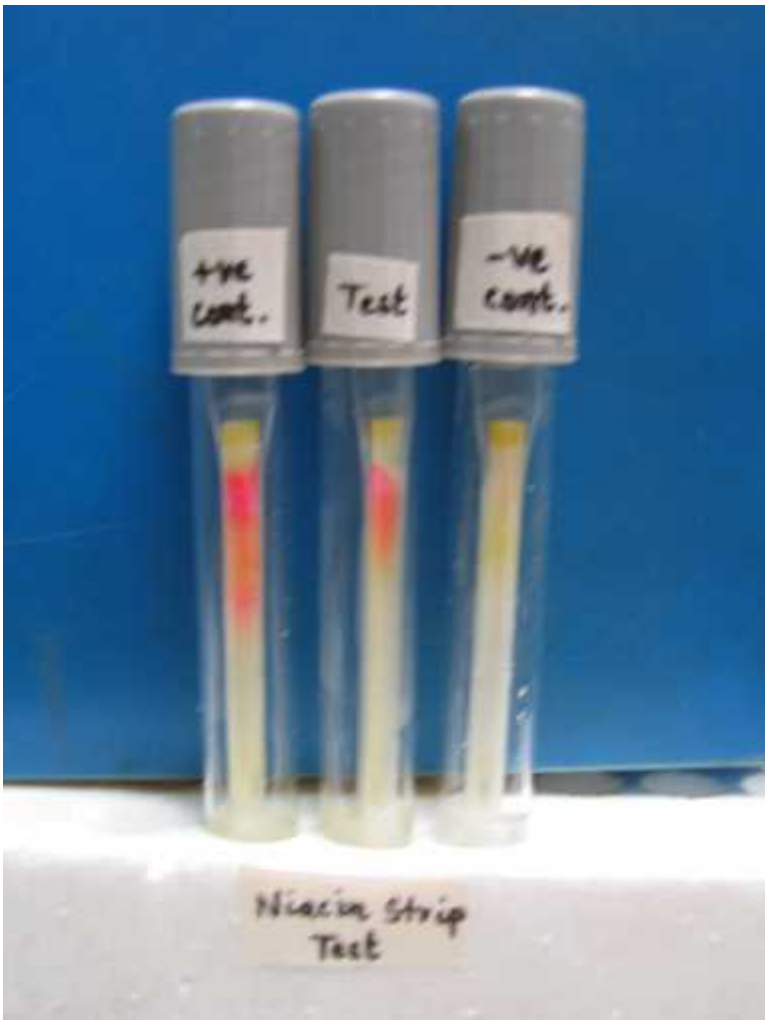


Photograph 6: *M. tuberculosis* growth on culture tubes according to decontaminating reagents



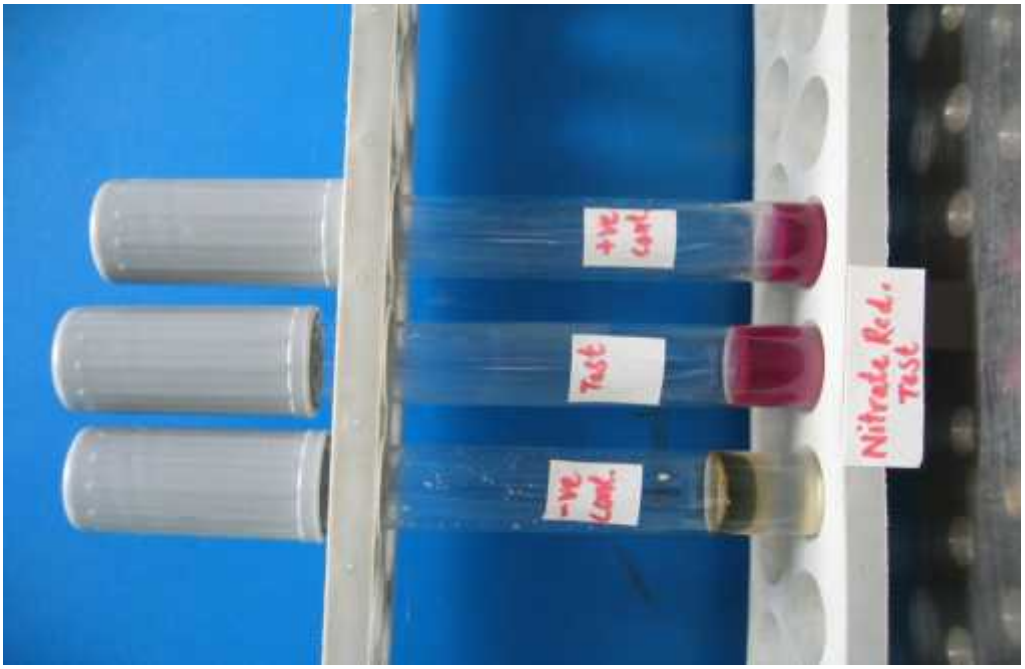
Photograph 7: Heat labile catalase test at 27°C and 68°C

Photograph 8: Niacin testing Strip



Photograph 9: Niacin Strip test

Photograph 10: No growth observed in PNB containing medium



Photograph 11: Nitrate reduction test

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The rapid detection and identification of mycobacteial pathogens in sputum samples are essential for effective treatment of tuberculosis. In the present study, the sputum samples were subjected to fluorochrome staining for detection of the acid-fast bacilli, followed by decontamination of the sputum samples by two different decontamination techniques i.e. Petroff's method and Nekal Method; and cultured on MOG, MLJ and newly proposed BLJ media for the detection of *M. tuberculosis*. The positive, negative and the contamination rates of the growth in culture media were compared among different cultural media and decontamination methods.

The rapid diagnosis of mycobacterial disease relies primarily on the detection of acid-fast bacteria by microscopy; however, it requires about 5,000-10,000 bacteria per ml of sputum and is considered nonspecific for species identification and differential diagnosis. Cultural method helps to identify the infecting species, more effectively.

To the best of our knowledge this study is the first of its kind in which the MLJ has been modified by addition of monopotassium phosphate to make the concentration of monopotassium phosphate in it to 2%, and evaluated it with the MLJ and MOG, for culture of *M. tuberculosis*.

The efficiency and the sensitivity of cultural technique over the microscopic smear observations are evident. The most widely used solid cultural media for the culture of *M. tuberculosis* are MLJ and MOG. Studies have shown that the efficiency of MOG is greater than that of MLJ as it gives more percentage of positive result than MLJ. However, MLJ has been widely used all over the world and has been recommended by IUATLD (Hans *et al.*, 1998), because of its high predictive value than Ogawa. MOG needs more amount of egg homogenate (approximately 2000ml for 1000ml of salt solution), than that for MLJ (approximately 1610ml for 1000ml of salt solution) (WHO, 1998b). The study tries to investigate the performance of MLJ by increasing the amount of monopotassium phosphate buffer in it. The study also tries to compare the efficiency of MOG, MLJ and BLJ media.

The efficiency of MLJ may be made comparable to MOG in two ways:

I. By increasing the content of the egg homogenate in MLJ to make it 2000ml for 1000ml salt solution. This would be non desirable and non economic as it would add extra cost to the media.

II. By increasing the amount of the potassium mono phosphate buffer.

MOG contains 2% monopotassium phosphate buffer while MLJ contains only 0.4% monopotassium phosphate buffer. Thus by increasing the amount of the monopotassium phosphate buffer in MLJ to make it 2% we get a comparative study.

Secondly, this study tries to compare the two decontamination techniques for sputum samples i.e. Petroff's method (which uses 4% NaOH, without need of neutralization and centrifugation) and Nekal method (which uses Nekal-BX, and needs overnight incubation and centrifugation facilities). The Nekal method of decontamination is believed to be more efficient.

In this study, in Petroff's method, the sputum samples were directly inoculated into MLJ, BLJ and MOG media after their decontamination with 4% NaOH, so as to make the comparison of BLJ easier with MOG which does not need neutralization and centrifugation procedure as required by MLJ.

If MLJ could be made as culture positive as MOG and use it without the need of neutralization and centrifugation, it would be benificial for the developing countries like Nepal. Again, the comparative study of the decontamination techniques would suggest which technique is more efficient and will save time and money by avoiding the problem of the repeating culture.

6.1.1 Study Group A

A total of 207 (94.09%) smear positive samples were found to be positive in one or all Culture media, while the remaining were negative. The culture negative result of the smear positive samples may either be due to environmental mycobacteria/MOTT or the dead myocbacteria, or other impurities that obtained AFB staining, or due to the killing of the bacteria during decontamination procedure.

6.1.1.1 Comparison of positive rates among culture media

6.1.1.1.1 Comparison between MLJ and MOG:

When comparing the results of total of 440 tubes of MLJ and MOG; MOG gave 92.05% positive result while MLJ gave only 84.32% positive result. The result was significant at 99% level of significance. In this study, the results from samples decontaminated by Nekal and 4% NaOH were pooled together to determine the total positive results.

When comparing the above result individually on the basis of decontaminating agent; in those decontaminated by 4% NaOH, MOG had 92.73% positive result while MLJ gave only 81.3% positive result. Similarly, when comparing on the basis of Nekal, MOG had 92.73% positive result while MLJ gave only 87.27 % positive result. The result highlights that MOG gives higher culture positive results, no matter which decontaminating agent is used among 4% NaOH and though lesser by Nekal.

The results are in favor of the results from various researchers comparing the Lowenstein-Jensen medium and Ogawa medium using different decontaminating agent.

In a study conducted by Pauwels P *et al.*, taking 392 smear positive samples they obtained 95% positive result in 1% Ogawa versus 88.4% on Lowenstein-Jensen. (Pauwels *et al*., 1990).

In a study conducted by Mendoza MT *et al*., 1994, the comparison was made between the 128 samples, with parallel Ogawa and LJ cultures that completed incubation without contamination. Mycobacterial growth *was* noted to be 123 (96%) on Ogawa and 121 (92%) on LJ culture tubes. (Mendoza *et al.*, 1994).

In a study conducted by David *et al.*, among total of 276 samples, he found 33.7% positive on LJ while 35.5% were positive on Ogawa. (David *et al*., 2004)

In a study carried for the identification of *M. ulcerence* by Dorothy Yeboah-Manu *et al*, 2004, consisting of 164 samples, 68.3% was found positive on LJ with Glycerol and 53.6% was positive on Ogawa with glycerol (Dorothy *et al*, 2004).

From these observations, we may conclude that it is a general trend that Ogawa medium gives more positive result than Lowenstein-Jensen medium, which has also been fulfilled in the present study.

6.1.1.1.2 Comparison between MLJ and BLJ:

When comparing the total result of 440 tubes of MLJ and BLJ, 92.50% samples were positive on BLJ while only 84.32 % were positive on MLJ. The result was statically significant at 99% level of significance.

The increase in the positive results may be due to the increase in the buffering capacity of the BLJ as it contains 2% monopotassium phosphate bffer in compare to 0.4% in MLJ. Thus one important inference that can be drawn from this result is that, the positive result was increased when BLJ was used in place of MLJ. So, it is better to use BLJ in place of MLJ if possible.

6.1.1.1.3 Comparison between MOG and BLJ:

When comparing the total result of 440 tubes of MOG and BLJ; 92.05% samples were positive on MOG while 92.50 % were positive on BLJ. The difference was not found to be significant.

The result clearly indicates that newly prepared BLJ is as efficient as MOG in terms of providing positive result.

The increase in positive result in MOG and BLJ than in MLJ may be due to the increased amount of buffering capacity of these media which facilitates the growth of *M. tuberculosis* present in the sample by neutralizing the toxic effect of the decontaminating agents used for decontamination of the sputum samples. The result also emphasizes the observation that by addition of monopotassium phosphate in MLJ to make it its concentration 2%, the rate of positive result also increases even slightly more than that of MOG. These were the findings the present study intended to uncover.

6.1.1.2 Comparative recovery of bacteria in different culture media with decontamination by Nekal and 4%NaOH:

6.1.1.2.1 Based on results of MLJ:

The difference in positive rates among 4% NaOH (81.3%) and Nekal (87.27%) for MLJ was significant at 95% level of significance. This result indicates that the Nekal method of decontamination yields higher positive result than 4% NaOH for MLJ.

The greater number of positive result in MLJ decontaminated with Nekal, than those decontaminated with 4% NaOH may be due to the fact that, the harsh chemical property

of 4% NaOH, being a hard decontaminating agent, kills most of the mycobacteria present in sputum sample along with the other contaminants during the decontaminating procedure. Slight increase in the decontamination timing (recommended is 15 to 20 minutes) (WHO, 1998b) would result in higher number of culture negative results. Nekal being a soft decontaminating agent which needs 16-18 hours of incubation to effectively kill the contaminating agents, the more resistant mycobacteria are virtually less harmed. At the same time, the mycobacteria are accumulated in the sediment by precipitation in the presence of barium, calcium, and phosphate ions (Warlo and Doerr, 1976).

6.1.1.2.2 Based on results of MOG and BLJ:

The difference in positive rates among samples decontaminated by 4% NaOH (91.36%) and Nekal (92.73%) and inoculated on MOG was not significant at 95% level of significance. Similarly, difference in positive rates among samples decontaminated by 4% NaOH (92.27%) and Nekal (92.73%) and inoculated on BLJ was also not significant at 95% level of significance.

This result indicates that the use of either 4% NaOH or Nekal as decontaminating agent does not affect the appearance of positive result on MOG and BLJ and thus any one of them may be used with same efficiency. The similar culture positive results in MOG and BLJ may be due to the fact that they have more buffering capacity (due to the presence of 4% potassium mono phosphate buffer) than MLJ (with 0.4% potassium mono phosphate buffer) and are able to neutralize the mycobactericidal effect of the 4% NaOH more effectively, while Nekal being a soft agent remains unaffected.

The Nekal method of decontamination is mostly used in European countries and there has not been any comparison between Preoff's (4% NaOH) method and Nekal-BX method in my knowledge. Most of the studies on Nekal-BX have been carried out in Germany and other European countries, thus most of the literatures were in German language and only a few authors translation of abstracts were available.

In a study carried out by Koch H *et al*, 1975, 91% of the sputum samples were found positive when decontaminated by Prawozell NBX (Nekal-BX) while 83.7% positive result was obtained when sputum samples were homogenized with Ditalan (Koch H *et al*, 1975). Similarly, in another study carried out by Koch H *et al*, 1977, 85.7% of the

samples were found positive when decontaminated by Prawozell NBX (Nekal-BX) while 89% positive result was obtained when sputum samples were homogenized with Ditalan (Koch H *et al*,1977).

6.1.1.3 Comparison of negative and Contamination rates:

For an ideal culture media, it is highly important that the media give as much less negative result and less contamination as possible to avoid the unnecessary repetition of culturing. The present study also intended to study the contamination rates and negative result among the three media compared.

6.1.1.4 Negativity rate:

The negativity rate was highest in MLJ with 4% NaOH as decontaminating agent (12.27%), followed by MLJ with Nekal as decontaminating agent (5.54%). When compared among BLJ and MOG the negative rate was higher in MOG (6.36% in 4% NaOH and 4.55% in Nekal) than in BLJ (4.55% in 4% NaOH and 4.09% in Nekal). The result indicates that BLJ is the most efficient media in terms of giving low negative results among smear positive samples followed by MOG and least efficient was MLJ.

The difference in negativity rates among 4% NaOH (12.27%) and Nekal (5.54%) for MLJ was significant at 95 % level of significance. This result indicates that the Nekal method of decontamination yields less negative result and is more efficient than 4% NaOH for MLJ.

The difference in negative rates among samples decontaminated using 4% NaOH (6.36%) and those using Nekal (4.55%) for MOG was not significant at 95 % level of significance. Similarly, difference in negative rates among 4% NaOH (4.55%) and Nekal (4.09%) for BLJ was also not significant at 95 % level of significance. This result indicates that the use of either 4% NaOH or Nekal does not affect the appearance of positive result and thus any one of them may be used with same efficiency for MOG and BLJ.

These results may be explained by pointing out the fact that 4% NaOH is a hard decontaminating agent having harsh chemical property, which kills greater number of *M. tuberculosis* along with other contaminants, but Nekal being soft reagent virtually kills less number of *M. tuberculosis* and yields higher positive result. (Collins *et al.*,

1997; Kent and Kubica, 1985). Similarly, MOG and BLJ having greater amount of monopotassium phosphate buffer (2%) counteracts the toxic effect of 4% NaOH when the decontaminated sample is inoculated into these media, thus neutralize the toxic effect and gives higher positive result while MLJ containing less amount of monopotassium phosphate buffer (0.4%) does not have enough buffering capacity to neutralize the toxic property of 4% NaOH, thus yielding less positive results. Due to this problem earlier Petroff's methods used HCl to neutralize the toxic effect of 4% NaOH (WHO, 1998b) and centrifugation (F. Ang *et al.*, 2001).

6.1.1.5 Contamination rate:

The contamination rate was highest in MLJ decontaminated by Nekal (7.27%) followed by MLJ decontaminated by 4% NaOH (6.36%). When comparing among BLJ and MOG, the contamination was higher in BLJ (3.18% in 4% NaOH and 3.18% in Nekal) than in MOG (2.27% in 4% NaOH and 2.73% in Nekal).

Since Nekal is soft decontaminating agent and needed 16-18 hours of incubation, the microorganisms which could survive these conditions and may have caused the contamination. Most of the contaminants were found to be fungi which could survive these harsh conditions. The higher contamination in MLJ may be explained on the fact that due to the harsh chemical action of the 4% NaOH, most of the *M. tuberculosis* were killed and those survived took some time to recover and grow on MLJ; due to which the contaminants like fungi had better chance to utilize the media and survive. The result indicates, though the contaminant rate is greater in BLJ than MOG and highest is in MLJ.

The result is in favor of the study conducted by Mendoza Myrna T., 1994, in which 6% of the 325 specimens in both LJ and Ogawa tubes were contaminated. Four percent more of the LJ tubes got contaminated during incubation (8 weeks). Only one Ogawa tubes got contaminated during incubation (Mendoza Myrna T., 1994).

The result may be explained by the fact that MOG contains higher amount of 2% malachite green than MLJ, which prevented greater amount of contaminating microorganisms to grow on MOG while these contaminating agents had optimum chance to grow on MLJ and BLJ. It is also important to note that a laboratory which experiences no contamination is probably using a method that kills too many of the

tubercle bacilli and generally a contamination rate of 2-3% is acceptable in laboratories (Collins *et al.*, 1997; Karin *et al*., 2006b).

In a study carried out by Pichula K *et al*., 1981, where a comparative study of different homogenization techniques using detergents: Chlorhexidinum gluconicum, Ditalan WO hc, Sodium-Laurylsulphate, Daurosept and Nekal BX, the lowest percentage of contamination was observed after homogenization by Nekal BX (Pichula K *et al*., 1981).

6.1.2 Study Group B

6.1.2.1 Comparison among smear negative samples

This study also studied the positive, negative and contamination rates among the smear negative samples to find out the rates at which the different media provided smear negative culture positive results.

Out of 104 smear negative samples studied, 9.62% (10/104) were found to be positive by one or all culture, the remaining 91.35% (95/104) were negative, and none of the samples were contaminated in all six tubes. The finding emphasizes the superior sensitivity of cultural techniques over the microscopy. The culture positive result of the microscopy negative samples may be due to the fact that the organisms were not well stained during the staining procedure or were in very less number to be seen during microscopic observation or they escaped the eyes of the inspection staff.

The highest smear negative culture positive result was given by MLJ (5.77%) and BLJ (5.77%) when Nekal was used as decontaminating agent; followed by MOG (4.81%) with 4% NaOH as decontaminating agent; then MOG (3.85%) and BLJ (3.85%) with 4% NaOH as decontaminating agent; and finally by MLJ using 4% NaOH as decontaminating agent (2.88%). The result showed that the use of Nekal as decontaminating agent increases the probability of finding smear negative culture positive result than 4% NaOH.

The result may be due to the fact that the specimens containing very low number of acid-fast bacteria were missed during the microcopy examination, but were preserved during the treatment by Nekal, thus giving greater positive result and those decontaminated using 4% NaOH were killed and yielded less number of positive result.

The above result also indicates that the use of BLJ or MOG increases the chance of finding smear negative culture positive than using MLJ. This may be due to higher buffering capacity of the BLJ and MOG which neutralizes the toxic effect when 4% NaOH is used as decontaminating agent, and facilitates the growth of the *M. tuberculosis* (Kent and Kubiac, 1985).

Most of the culture positive results were obtained on 3rd, 4th and 5th weeks. This indicated that there were no rapid growers in the specimen (Kent and Kubiac, 1985).

All the culture positive results were confirmed for their acid fastness by Ziehl-Nelsen Staining. 20% (44 tubes) of total culture positive samples were biochemically tested by p-Nitrobenzoic Acid (PNB) test, catalase test, Nitrate reduction test, Niacin strip tests. The samples for the biochemical tests were selected by random sampling and all of the tested samples gave positive results for *M. tuberculosis* (Kent and Kubiac, 1985).

The appearance of positive result during culture of *M. tuberculosis* depends upon different factors such as:

- concentration of decontaminating agent used
- time of Centrifugation during the concentration procedure
- buffering capacity of the media
- proper handling of the specimens
- types of contaminants present in the sputum sample (Collins *et al.*, 1997).

The culture negative result of smear positive samples may be due to:

- acid fast bacilli seen under microscope had lost their ability to grow on culture medium
- in patients relieving chemo-therapy, the organisms may have lost their ability to grow on culture media and be particularly dead
- sputum specimens may have been exposed to sunlight or heat, dried out or contaminated
- excessive decontamination procedures before inoculation
- inadequate culture media

- tubercle bacilli were not evenly distributed in sputum specimen
- in staining process, tap water used for washing of slide may contain saprophytic acid fast bacilli which contaminated the slide during washing step giving smear positive result (Collins *et al.*, 1997).

In conclusion, BLJ proposed in this study is closely comparable with MOG and it allows direct identification of *M. tuberculosis* in sputum samples.

Due to high positive result than in MLJ even when 4% NaOH is used as decontaminating and homogenizing agent, and excludation of neutralization and centrifugation steps, the newly proposed BLJ can be highly useful for the low economy Laboratories of developing countries using MLJ for culture and having facilities of sample processing and culture incubation. Due to the increased buffering capacity of BLJ, slight alteration in recommended 15 to 20 minutes of decontamination timing for Petroff's method would not have profound impact on the positive result which is also an advantage for countries like Nepal where highly trained manpower are few.

6.2 Conclusion

Hence, it is concluded that media prepared by addition of mono potassium phosphate buffer to the Modified Lowenstein-Jensen media, to make the concentration 2% (BLJ), yields higher positive result from smear positive samples than Modified Lowenstein-Jensen media itself; and the media thus made gives as much positive result as MOG does. Similarly, the study also found that the use of Nekal method of decontamination yields more positive result than 4% NaOH, if the media used for the culture is Modified Lowenstein-Jensen media; while the use of either Nekal or 4% NaOH for Modified Ogawa and BLJ does not yield significant difference in terms of positive results. Thus the use of BLJ using 4% NaOH as decontaminating agent is highly recommendable for the developing countries like Nepal.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

- In this study, monopotassium phosphate buffer concentration of MLJ was increased to 2% (BLJ) and evaluated it with the MLJ and MOG, for detection of *M. tuberculosis* in sputum samples.
- Simultaneously, modified Petroff's method and Nekal-BX method of decontamination were compared to evaluate their effectiveness.
- A total of 324 samples, 220 smear positive and 104 smear negative were selected.
- Out of 220 smears positive samples, 94.091% were found to be positive by one or all culture and out of 104 smear negative samples, 9.62% were found to be positive by one or all culture.
- Among smear positive sputum samples decontaminated using Petroff's method, 91.36%, 81.36% and 92.27% samples were culture positive on MOG, MLJ and BLJ, respectively; and those decontaminated using Nekal method, 92.72%, 87.73% and 92.72% samples were culture positive on MOG, MLJ and BLJ, respectively.
- Among smear negative sputum samples decontaminated using Petroff's method, 3.85%, 2.88% and 3.85% samples were culture positive on MOG, MLJ and BLJ, respectively; and those decontaminated using Nekal method, 4.81%, 5.77% and 3.85% samples were culture positive on MOG, MLJ and BLJ, respectively.
- MOG yielded more positive result (92.05%) than MLJ (84.32%) ($P<0.01$). BLJ yielded more positive result (92.50%) than MLJ (84.32%) ($P<0.01$). MOG and BLJ yielded similar positive result 92.05% ($P>0.05$).
- The samples decontaminated by Nekal yielded better result (87.27%) than those decontaminated by 4% NaOH (81.3%) for MLJ ($P<0.05$). There was no significant difference in the culture positive results among samples decontaminated by 4% NaOH (91.36%) and Nekal (92.73%) cultured on MOG

(P>0.05); and BLJ gave 4% NaOH (92.27%) and Nekal (92.73%) culture positive results(P>0.05).

- The highest smear negative culture positive result was given on MLJ (5.77%) and BLJ (5.77%) with decontamination by Nekal.

7.2 Recommendations

Based on the finding of this study, the following recommendations have been made:

- The efficiency of commercially available MLJ may be enhanced by increasing its monopotassium phosphate buffer level to 2% to it to get higher positive result. The media thus prepared yield positive result similar to MOG.
- If the culture of *M. tuberculosis* is being carried out using MLJ, it is better to process the sputum sample by Nekal as than 4% NaOH and any one of them may be used if MOG or BLJ is being used.
- The use of BLJ should be started in the laboratories which are presently using MLJ; for this method the steps of neutralization and centrifugation are not needed and low economy microbiology laboratory having sputum processing and culturing facilities can effectively use this method.
- The data obtained from the present study may be used for the further analysis of different aspects of BLJ; such as rate at which 3+, 2+, 1+, and few bacilli detected by microscopy are recovered by BLJ.
- The present study was based only on total of 324 samples (220 smear positive and 104 smear negative samples); further study with larger number of samples should be carried out in order to verify the results obtained from this study.

REFERENCES

Ahlburg D (2000) The economic impacts of tuberculosis. WHO (document WHO/CDS/STB/2000.5), htt://www.stoptb.org/conference/ahlburg.pdf

Akhtar M, Bretzel G, Boulahbal F, Dawson D, Fattorini L, Feldmann K, Frieden T, Havelkova M, Kantor IN, Kim SJ, Kuchler R, Lamothe F, Laszlo A, Casabona NM, Rieder HL, McDougall C, Miorner H, Orefici G, Paramasivan CN, Pattyn SR, Reniero A, Ridderhof J, Rusch Gerdes S, Siddiqi SH, Spinaci S, Urbanczik R, Vincent V and Weyer K (2000) Technical guide: sputum examination for tuberculosis by direct microscopy in low income countries. 5th edition, International Union against Tuberculosis and Lung Disease (IUATLD), 68 boulevards Saint-Michel, 75006 Paris, France.

Amatya NG (1992) National Tuberculosis Control Programme in Nepal and its progress in this year: Seminar and workshop on National Tuberculosis Control Programme. National Tuberculosis Centre, Thimi, Bhaktapur, Nepal.

American Thoracic Society and Center for Disease Control (1981) The tuberculin skin test. Am Rev Respir Dis. 124:356-363.

American Thoracic Society and Center for Disease Control (2000) Diagnostic standards and classification of tuberculosis in adults and children. Am J Respir Crit Care Med. 161:1376-95.

Ananthanarayan R and Panikar JCK (1996) Text book of microbiology. 5th edition, Orient Longman Limited, India, pp-324-337.

Ang F, Mendoza MT, Santos HR, Celada-Ong R, Enrile CP, Bulatao WC and Aguila AM (2001) Isolation Rates of Mycobacterium tuberculosis from Smear-negative and Smear-positive Sputum Specimen Using the Ogawa Culture Technique and the Standard Lowenstein-Jensen Culture Technique. Phil J Microbiol Infect Dis 30(2): 37-39.

Barez MYC, Mendoza TM, Celada RS and Santos HR (1995) Accuracy of AFB in relation to TB culture in detection of pulmonary tuberculosis. Phil J Microbiol Infect Dis 24:33-36.

Braksdale L and Kim DS (1977) *Mycobacterium*. Bacteriol Rev. 41:217-23-73.

Bass JB, Farer LS, Hopewell PC (1990) Diagnostic standards and classification of tuberculosis. Am Rev Respir Dis. 142:217-372.

Behr MA, Warren SA, Salamon H, Hopewell PC, Leon AP, Daley CL and Small PM (1999) Transmission of *M. Tuberculosis* from patient smear negative for acid fast bacilli. Lancet. 353:444-449.

Bhargava A, Jain A and Agrawal SK (2007) A comparison of Liquid and Solid culture media with radiometric system for detection of mycobacteria in clinical. Ind J Tub. 48:9.

Bhat KG and Bhat G (2000) Methods and devices in simple cold staining method for acid-fast bacilli. Trop Doct. 30:224.

Bloom BR (1994) Tuberculosis: pathogenesis, protection and control. American Society for Microbiology. Washington DC, pp-26-28

Braunstein H, Hicks L and Kony C (1990) *Mycobacterium africanum* and other members of tuberculosis complex. American Society of Clinical Pathology-Microbiology. MB 90-4, 33:1-5.

Brennan PJ and Draper P (1994) Ultrastructure of *Mycobacerium tuberculosis*: Pahogenesis, Protection, and Control, (ed.) Bloom BR, American Society for Microbiology Press, Washington, DC, pp 271-284.

Brodie D and Schlunger NW (2005) The diagnosis of tuberculosis. In: Schlunger NW (eds) Clinics in Chest Medicine, Tuberculosis. Clin Chest Med. 26(2):247-271.

Brooks JB, Daneshvar MI, Fast DM and Good RC (1987) Selective procedures for detecting femtomole quantities of tuberculostearic acid in serum and cerebrospinal fluid by frequency-pulsed electron-capture gas-liquid chromatography. J Clin Microbiol. 25:1201-1206.

Chakraborty P (2003) A textbook of microbiology. 2nd edition., New Central Book Agency (P) LTD. 8/1. Chintamoni Das Lane, Kolkota 700 009, India, pp 396-408.

Cheesbrough M (2000) Medical laboratory manual for tropical countries, Vol. 2, Microbiology, ELBS, pp 289-299.

Chonde T, Cruz R, Kantor IND, Jain NK, Laszlo A, Latini O, Urbanczik R and Wright P (2000) International course on the management of tuberculosis laboratory networks

in low-income countries. International Union against Tuberculosis and Lung Disease (IUATLD), World Health Organization (WHO), Health Canada. 2-13 Ottawa, Canada.

Collins CH, Grange JM and Yates MD (1984) A review: mycobacteria in water. Journal of Applied Bacteriology. 94:135-149.

Collins CH, Grange JM and Yates MD (1997) Tuberculosis bacteriology: Organization and practice. 2nd edition, Oxford, Butterworth-Heinemann, pp 1-80.

Dalovisio JR, Montenegro JS and Kemmerly SA (1996) Comparison of amplified *Mycobacterium tuberculosis* (MTB) direct test, Amplicor MTB PCR, and IS*6110*-PCR for detection of MTB in respiratory specimens. Clin. Infect. Dis. 23 (5): 1099-106.

David HL, Jahan MT and Jumin A (1978) Numerical taxonomy analysis of *Mycobacerium africanum*. Int J Syst Bacteriol. 28: 464-73.

David L, Habeenzu C and Mitarai M (2004) Evaluation of an Ogawa Mycobacterium culture method modified for higher sensitivity employing concentrated samples. The Japanese Society of Tropical Medicine Tropical Medicine and Health. 32(1):1-4.

Des Prez and Heim CR (1991) Mycobacterium tuberculosis, in Principle and Practice of Infectious Disease. 3rd edition, Churchill Livingstone, New York, pp 265-281.

Difco/BBL Product Information: Lowenstein Media, Lowenstein Medium Base, Lowenstein-Jensen Medium, Lowenstein-Jensen Medium, Gruft Lowenstein-Jensen Medium with Iron, Lowenstein-Jensen Medium with Pyruvic Acid, Lowenstein-Jensen Medium with 5% Sodium Chloride, pp 315-138.

Dixon and Cuthbert (1967) Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. Am Rev Respir Dis. 96:119.

Dorothy Yeboah-Manu, Thomas Bodmer, Ernestina Mensah-Quainoo, Samuel Owusu, David Ofori-Adjei and Gerd Pluschke (2004) Evaluation of Decontamination Methods and Growth Media for Primary Isolation of Mycobacterium ulcerans from Surgical Specimens, Journal Of Clinical Microbiology. DOI: 10.1128/JCM.42.12, pp 5875–5876.

Dubos T and Dubos J (1953) The white plague: Tuberculosis man and society. J Hist Med Allied Sci. 43:165-82.

Dye C (2000) Tuberculosis 2000-2010: Control, but not elimination. Int J Tuberc Lung Dis. 4(12):S146-S152.

Dye C, Scheele S, Pathani V and Raviglione MC (1999) Global burden of tuberculosis estimated incidence, prevalence and mortality by country. JAMA. 282:677.

Eiken Genome Site (2005) The principle of LAMP method. Eiken Chemical Co. Ltd. http:// eiken.co.jp

Enarson DA, Rieder HL, Arnadottir T and Trebucq A (2000) Management of tuberculosis: a guide for low-income countries, 5th edition. International Union against Tuberculosis and Lung Disease (IUATLD), 68 boulevards Saint-Michel, 75006 Paris, France.

Forbes BA, Sahm DF and Weissfeld AS (2002) Bailey and Scott's Diagnostic Microbiology. 11th edition, Mosby, Inc.USA, pp 265-278.

Foulds J and O'Brien R (1998) New tools for the diagnosis of tuberculosis, the perspective of developing countries. Int. J. Tuberc Lung Dis. 2(10):778-783

Gobin J and Moore CH (1995) Iron acquisition by *Mycobacterium tuberculosis*: isolation and characterization of a family of iron-binding exochelins. Proc Natl Acad Sci USA. 92: 5189-93.

Gaensler EA (1982) The surgery for pulmonary tuberculosis. Chest. 1255:73-84.

Good RC and Shinnick TM (1998) Mycobacterium. In: Collier L, Balows A and Sussman M (eds) Topley and Wilson's Microbiology and Microbial infection, Systematic Bacteriology. Vol. 2, 9th edition, Edward Arnold, London, United Kingdom, pp-536-582.

Grange JM (1990) Tuberculosis: In M. T. Parker, B. I. Duerden, (ed.), Topley and Wilson's Principle of Bacteriology, Virology and Immunity. Vol. II. 8th edition, Butler and Tanner Ltd, London, pp 93-118.

Grange JM (1996) Microbacteria and Human Disesase. 2nd edition, Arnold publication, London, pp 56-78.

Greenwood D, Slack RCB and Peutherer JF (2002) Medical microbiology. 16th edition, Churchill Livingstone, UK pp 200-213.

Gross W and Wayne, LG (1970) Nucleic acid homology in the genus Mycobacterium. Am. Rev. Respir. Dis. 45:232

Gruft (1963) Addition of ribonucleic acid in LJ. Am. Rev. Respir. Dis. 88:412.

Gruft (1971) Addition of Penicillin to LJ. Health Lab. Sci. 8:79.

Hal RM, Grinathanran M, Messanger AJM and Rotledge C (1987) Iron transport in Mycobacterium semegmatis. Am Rev Respir Dis. 12:15-57.

Hal RM and Ramakrishna T (1977) Rate of ribonucleic growth in Mycobacterium tuberculosis H37. J Bacteriol. 129:616-22.

Hans LR, Chonde TM, Myking H, Urbanczik R, Laszlo A, Kim SJ, Deun AV and Trébucq A (1998) Tuberculosis Reference Laboratory and the National Laboratory Network, Minimum Requirements, Role and Operation in a Low-Income Country National Laboratory Network. International Union Against Tuberculosis and Lung Disease (IUATLD) 68, boulevard Saint-Michel, 75006 Paris, France.

Heifetes LB and Good RC (1994) Current laboratory methods for the diagnosis of tuberculosis. In: Bloom BR (eds) Tuberculosis, pathogenesis, protection and control. ASM Press, Washington, DC, pp 49-88.

Henry JB (1991) Clinical Diagnosis Management by Laboratory Method. Journal of Clinical Microbiology. 21: 721-725.

Huebner RE, Schein MF and Bass JB (1993) The tuberculin skin test. Clin. Infect. Dis. 17: 968-975.

Hughes (1966) Addition of Pyruvic acid to egg based media. J. Clin. Pathol. 19:73.

Ichiyama S, Shimokata K, Takeuchi J and the AMR Group (1993) Comparative study of a biphasic culture system (Roche MB check system) with a conventional egg medium for recovery of mycobacteria. Medline. Tuberc. Lung Dis. 74: 338-341.

Indo-Asian News Service (2005) Tuberculosis kills 7,000 in Nepal annually, 25th March 2005. http//in.news.yahoo.com.

Iseman MD (1993) Treatment of multi-drug resistant tuberculosis. The New England Journal of Medicine. 329 (11):784-791.

Iwamoto T, Sonobe T and Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbial. 41(6):2616–2622.

Johansen IS (2006) Rapid diagnosis of mycobacterial diseases, and their implication on clinical management. Danish Medical Bulletin. 53:28-45.

Joklik WK, Willet HP, Amos BD and Wilfert CM (eds) (1992) Zinsser Microbiology. 20th edition. Prentice Hall International Inc, pp 497-510.

Kar A, Parekh K and Chakraborty AK (2003) Advances in tuberculosis diagnostics. Health Administrator. 15:118-123.

Karin W, Roxanna R and Paul VH (2006a) Southern Africa Health info Tuberculosis, Part II: Culture homogenisation and decontamination. SAHealthInfo. http//:www.sahealthinfo.org/tb/tb.htm.

Karin W, Roxanna R and Paul VH (2006b) Southern Africa Health info Tuberculosis, Part II: Culture homogenisation and decontamination. SAHealthInfo. http//:www.sahealthinfo.org/tb/tb.htm.

Keers RY (1978) Pulmonary Tuberculosis: A Journey down the Centuries. Bailliere tindall publication, London, pp 159-178.

Kent PT and Kubica GP (1985) Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease control, Atlanta, pp 1-91.

Kilburn JO and Kubiac GP (1968) Reagent-impregnated paper strips for detection of niacin. Am Med J Clin Pathol. 50:530-2.

Koch H, Gerloff W, Kalich B and Uhlemann KR. (1975) Homogenization of the specimens for culturing mycobacteria by means of the detergents Präwozell N-BX and ditalan E (author's transl). 143(3):212-8. http//www.ncbi.nlm.nih.gov

Koch H, Rennert G, Gerloff W, Hergt R, Kalich R, Knauf B, Koedel H and Uhlemann R (1977) Comparative study between the surface-active substances prawozell NBX (Nekal BX) and Ditalan WO hc for homogenization and decontamination of

specimens for the cultivation of mycobacteria (author's transl). 148(2):227-35 http//www.ncbi.nlm.nih.gov

Kox LFF (1996) Diagnosis of tuberculosis and other mycobacterioses: development and clinical evaluation of PCR assay. A thesis of department of biomedical research, Royal Tropical Institute, Amsterdam and Division of Pulmonary Disease, Academic Medical Center, Amsterdam, pp 45-126.

Levinson WE and Jawetz E (eds) (1992) Medical Microbiology and Immunology, Mycobacteria. 2nd edition, Appleton & Lange, East Norwalk. CT, pp 113-126.

Lowell AM(1966) A view of tuberculosis morbidity and mortality fifteen years after the advent of the chemotherapeutic era 1947-1962. Adv Tuberc Res. 15:55-124.

Medigan MT, Martinko JM and Parke J (2000) Brock Biology of Microorganisms, 9th edition, Prentice Hall International, Inc. USA, pp 496-226.

McMurray DN (2001) Baron's medical microbiology textbook. Online textbook. http://gsbs.utmb.edu/microbook/toc.htm

Mehra NK and Rajalingam R (2001) Susceptibility facrors in pulmonary tuberculosis:special emphasis on HLA. In: Sharma SK and Mohan (eds) Tuberculosis. Medical publishers (p) Ltd, New Delhi, pp14Mendoza MT, Santos HR, Celada RS, Ang CF, Sombrero LT (2004) Comparison of the efficacy of Ogawa culture medium and Lowenstein-Jensen medium for culture isolation of Mycobacterium tuberculosis. http//www.herdin.ph

Mendoza MT, Santos HR, Celada RS, Ang CF and Sombrero LT (1994) The TB Study Group: Comparison of the efficacy of Ogawa culture medium and Lowenstein-Jensen medium for culture isolation of Mycobacterium tuberculosis. http//www.herdin.ph

Minnikin DE (1982) The biology of the mycobacteria. Ratledge C and Stanford JL (eds). Vol.1. Academic Press, New York, pp 469-553.

Morgan MA., Horstmeier CD, DeYoung DR and Robers GD (1983) Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. J. Clin. Microbiol. 18: 384-388.

Murray SJ, Barrett A, Magee JG and Freeman R (2003) Optimization of acid fast smears for the direct detection of mycobacteria in clinical samples. J Clin Pathol. 36:613-615.

National Tuberculosis Centre (NTC) (2001/2002) Annual Report. National tuberculosis control programme, HMG, Nepal, pp 7.

National Tuberculosis Centre (NTC) (2002/2003) Annual Report. National tuberculosis control programme, HMG, Nepal, pp 5.

National Tuberculosis Control Programme (NTP) (2002) TB facts, figures and concepts. Nepal, HMG, Ministry of Health, Department of Health Services.

National Tuberculosis Control Programme (NTP) (2004) Annual report of National Tuberculosis Control Programme Nepal. HMG, Ministry of Health, Department of Health Services.

National Tuberculosis Control Programme (NTP) (2006) Annual report of National Tuberculosis Control Programme Nepal. HMG, Ministry of Health, Department of Health Services.

Niemann S, Harmsen D, Rüsch-Gerdes S and Richter E (2000) Differentiation of clinical Mycobacterium tuberculosis complex isolates by gyrB DNA sequence polymorphism analysis. J. Clin. Microbiol. 38: 3231-3234.

Noordeen SK and Godal T (1988) Introduction. In: Rees RJW (eds) Tuberculosis and Leprosy. British Medical Bulletin 44(3): 523-527.

Park K (2000) Park's Textbook of Preventive and Social Medicine. 15th edition. M/s Banarsidas Bhanot Publishers, Jabalpur, India, pp 130-140.

Pauwels P, Kalonga MB, Sykalon SK, Willame JC and Carpels G (1990) Comparison of two culture media for the isolation of Mycobacterium tuberculosis. Ann Soc Belg Med Trop, Kinshasa, Zaire. 70(3):237 – 41.

Petroff SA (1915) A new and rapid method for the isolsation and cultivation of tubercule bacilli directly from the sputum and feces. J Exp Med. 21:38-42.

Petran EL and Vera HD (1971) Media for selective isolation of mycobacteria. Health Lab Sci. 8:225-30.

Philipine council for health research and development (PCHRD) (2003) Ogawa Culture Technique. http//: www.pchrd.dost.ph

Pichula K, Szaro A, Janowiec M, Chomenko A, Gerloff W, Hejny M, Kalfin E, Kalich R, Kubín M, Makarevicz NM, Mezensky L, Popovic I, Turzova M, Uhlemann R and

Ulber H (1981) Comparative studies on detergents - chlorhexidinum gluconicum, ditalan wo hc, sodium-laurylsulphate, laurosept, nekal bx - used for homogenization of diagnostic specimens in the microbiological diagnostic of tuberculosis (author's transl).156(3):255-66. http//www.ncbi.nlm.nih.gov

Ponticeellio A, Perna F, Sturbenboom MCJM, Etiello IM, Bocchino M and Sanduzzi A (2001) Demographic risk factors and lymphocyte populations in patients with tuberculosis and their healthy contacts. Int J Tuberc Lung Dis. 5: 1148-1155.

Rao RN, Viswanathan R., Deshmukh M, Pamra SP, Sen PK, Berdia NL and Dingley HB (1981) Textbook of Tuberculosis. The Tuberculosis association of India, India, pp 496-562.

Ratledge C (1982) Nutrition , growth and metabolism. The Biology of Mycobacteria, vol. 1, (eds) Ratledge C, Standford JL, Academic Press, London, pp 286-312.

Rijal B, Rahman M and Bam DS (2002) Multi-Drug Resistant Tuberculosis: an overview of the SAARC Region. STC Newsletter, Vol. XII. No. 1 Jan-June: pp13-14.

Roberts GD, Koneman EW and Kim YK (1991) Mycobacterium In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Yolken RH (ed.) Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, DC.

Rossenblatt MB (1973) Pulmonary tuberculosis: evaluation of modern therapy. Bull NY Acad Med. 49:163-96.

Salfinger M and Kafader FM (1992) Mycobactericeae Prüfung biochemischer Merkmale von Mikroorganismen In: Burkhardt, F., [Hrsg]: Mikrobiologischer Diagnostik, George Thieme Verlag, Stuttgart, New York, pp 78-165.

Satish Gupte (1999) Short text book of Medical Microbiology. 7th Edition, Jaypee Brother Medical Publisher (P). Ltd. pp 264-283.

Savic B, Sjobring U and Wilkins S (1992) Evaluation of polymerase chain reaction, tuberculostearic acid analysis and direct microscopy for detection of Mycobacterium tuberculosis in sputum. J Infect Dis. 166:1177-80.

Schliesser T (1985) Mycobacterium. In: Blobel H, Schliesser T (Hrsg): Handbuch der bakteriellen Infektionen bei Tieren. Bd. V. 1. Aufl. Gustav Fischer Verlag. Stuttgart. 65:155-313.

Schluger NW (2003) The diagnosis of tuberculosis: what's old, what's new. Semin Respir Infect. 18(4): 241 - 248. [PubMed: 14679473].

Sharma SK and Mohan A (2001) Tuberculosis. Jaypee Brother Medical Publishers (P) Ltd. New Delhi, pp 11-29.

Silcox D, Good RC and Floyd F (1981) Lowenstein-Jensen media. J. Clin. Micobiol. 14:686.

Somoskovi A, Hotaling JE, Fitzgerald M, Donnel D, Parsons LM and Salfinger M (2001) Lessons from a proficiency testing event for acid-fast microscopy. Chest. 120: 250-257.

Sonnenwirth AC and Jarett L (1990) Gradwohl's clinical laboratory method and diagnosis. 8th edition, B.I. Publication, New Delhi, pp 1384-1386, 1698.

Sritharan M and Sritharan V (2000) Polymerase chain reaction in the diagnosis of tuberculosis. Indian Journal of Clinical Biochemistry. 15(suppl): 200-216.

Tatsuji O (1960) The comparative studies between NaOH method and neutralization method for isolation of tubercle bacilli. Kitasato Arch Exp Med. 33:1-4; 9-24.

Tenover H, Crawfor M and Huebner T (1993) The resurgence of tuberculosis in your laboratory. J Clin Microbiol. 31: 767-70.

Tulip Diagnostics, Mycobacterium Tuberculosis: AFB Staining, Culture and Sensitivity. http://www.tulipgroup.com

Wadee AA, Bating L and Reddy SG (1990) Antigen capture assay for detection of a 45-kilodalton *Mycobacterium tuberculosis* antigen. J. Clin. Microbiol. 28: 2786-91

Waksman SA (1964) The conquest of tuberculosis. Berkeley and Los Angeles: University: Universitu of California Press, USA, pp 89-116.

Warlo HJ and Doerr HW (1976) Use of nekal BX for pretreatment of clinical material before culture to diagnose tuberculosis (author's transl). Zentralbl Bakteriol. 234(3):393-406 [Pubmed].

Watt B, Rayner A and Harris G (1996) Mycobacteria. In: Collee JG, Fraser AG, Marmion BP and Simmons A (eds) Mackie and McCartney practical medical microbiology. 14th edition, International Student Edition, Churchill Livingstone pp 265-286.

Wayne and Doubek (1968) DNA Homology of *M. tuberculosis.* Appl. Microbiol. 16:925.

Wayne LG (1982) Microbiology of tuberculosis. Am Rev Respir Dis. 125:31-41.

Wheeler and Ratledge (1994) Metabolism of Mycobacterium tuberculosis, tuberculosis: Pathogenesis, Protection, and Control, ed. Bloom BR, American Society for Microbiology Press, Wasington, DC, pp 353-385.

WHO (1993) WHO declares tuberculosis a global emergency [press release]. World Health Organization, Geneva, Switzerland.

WHO (1997) TB: A clinical manual for South East Asia. World Health Organization, Geneva, Switzerland.

WHO (1998 a) Laboratory services in tuberculosis control. Part II: microscopy. World Health Organization, Geneva, Switzerland.

WHO (1998 b) Laboratory services in tuberculosis control. Part III: culture. World Health Organization, Geneva, Switzerland.

WHO (2000) Stopping tuberculosis. World Health Organization regional office for South-East Asia, New Delhi.

WHO (2002) Tuberculosis. WHO information fact sheets. 104:1-3.

WHO (2006) Tuberculosis. WHO information fact sheet 104:1-4.

WHO (2007) Tuberculosis. WHO information fact sheet 104:1-4.

WHO (2007) Global tuberculosis control: surveillance, planning, financing. WHO report (document WHO/HTM/TB/2007.376). World Health Organization, Geneva, Switzerland.

Wilson G and Miles A (1964) Topley and Wilson's Principles of Bacteriology and Immunology. Vol.1, 5th edition, Arnold Publication Ltd., London, pp 536-582.

Woods GL (2002) The mycobacteriology laboratory and new diagnostic techniques. Infect Dis Clin North Am. 16(1): 127 - 144. [PubMed: 11917810].

Woods GL and JA Washington (1987) Mycobacteria other than *Mycobacterium tuberculosis*: review of microbiologic and clinical aspects. Rev. Inf. Dis. 9:275-294.

Yeager HJ Jr., Lacy J, Smith L, and LeMaistre C (1967) Quantitative studies of mycobacterial populations in sputum and saliva. Am. Rev. Respir. Dis. 95: 998-1004 [Medline].

Zaher F and Marks J (1997). Methods and medium for the culture of tubercule bacilli. Tubercule. 58: 143-145.

Zimmerman MR (1979) Pulmonary and osseous tuberculosis in an Egyptian mummy, Bull NY .A Cad Med. 55:604-8.

APPENDIX-I

1. Materials and chemicals

A) Reagents/chemicals

Absolute ethanol
Auramine O
Basic fuchsin powder
Carbol fuchsin
Disodium phosphate (Na_2HPO_4)
Distilled Water
Egg
Ethyl alcohol
Glycerol
Hydrochloric acid (HCl)
30% hydrogen peroxide (H_2O_2)
Malachite green
Methylene blue
Nekal
N-naphthylethylene-diamine
Phenol solution
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)
Sodium Hydroxide (NaOH)
Sodium nitrate ($NaNO_3$)
Sodium phosphate (Na_3PO_4)
Sulfanilamide
Sulphuric acid
Tween 80

B) Glasswares

- Beakers
- Centrifuge tubes
- Conical flasks
- Culture tubes
- Glass rod
- Measuring cylinder
- Pasture Pipettes
- Screw-capped test tubes
- Pipettes
- Slides

C) Equipments

- Autoclave
- Biological Safety Cabinet, Class III type
- Centrifuge
- Coagulator
- Distilling apparatus
- Florescence microscope
- Incubator
- Olumpus microscope
- Refrigerator
- PH meter
- Vortex mixer

D) Miscellaneous

- Bacteriological loop
- Bunsen burner Cotton
- Cotton
- Forceps
- Gloves

Labeling stickers

Staining rack

Tissue paper

Tube holders

Tube rack

Appendix-II

2. Staining reagents preparation

A. Flurochrome stain

i) Auroamine O Flurochrome stain

Alchoholic Aramine solution

Ingredients	Composition
Auramine O	0.1 g
Ethyl alcohol, 95%	100 ml

Phenol liquid

Ingredients	Composition
Phenol	30 ml
Distilled water	870 ml

Dissolve phenol in water with gentle heating and take 30 ml phenol into a flask. Add auramine stain gradually and shake vigorously until dissolved then add distilled water and ethanol added. Filter and store in a dark bottle.

ii) Decolorizing agent

20% aqueous sulphuric acid solution

Ingredients	Composition
Concentrated sulphuric acid	200 ml
Distilled water	800 ml

Pour the required volume of the distilled water into an Erlenmeyer flask. Slowly add the required volume of the concentrated sulphuric acid, allowing it to flow along the side of the flask. The mixture will be heated up. Cool the mixture and shake well and store in the reagent bottle for further use. Never pour water into concentrated sulphuric acid-explosive spills may occur.

iii) Counter stain

1% Methylene blue (100 ml)

Ingredients	Composition
Methylene blue chloride	1 gm
Ethanol	10 ml
Distilled water, to make	100 ml

Place the required amount of the methylene blue into the volumetric flask containing the require amount of the ethanol. Shake well the contents until completely dissolved and add the distilled water to make the final volume upto100 ml.

B. Ziehl-Neelsen staining

i) Ziehl's carbol fuchsin

10% Basic fuchsin alcoholic stock (solution A)

Ingredients	Composition
Basic fuchsin powder	1 gm
Ethanol	10 ml

Place the required amount of the basic fuchsin in the volumetric flask containing the required amount of the ethanol. Shake well the contents until complete dissolution and leave it for overnight. Small quantities of this solution should be filtered prior to staining.

Aqueous phenol solution (Solution B)

Ingredients	Composition
Distilled water	85 ml
Phenol (liquid)	5 ml

Before adding water, liquefy the phenol crystal in the flask by gentle heating. To prepare the 1% Ziehl's carbol fuchsin working solution, mix 10 ml of solution A with 90 ml of solution B on the next day, filter and store for the further use.

ii) Decolorizing agent

20% aqueous sulphuric acid solution

Ingredients	Composition
Concentrated sulphuric acid	200 ml
Distilled water	800 ml

Pour the required volume of the distilled water into an Erlenmeyer flask. Slowly add the required volume of the concentrated sulphuric acid, allowing it to flow along the side of the flask. The mixture will be heated up. Cool the mixture and shake well and store in the reagent bottle for further use. Never pour water into concentrated sulphuric acid-explosive spills may occur.

iii) Counter stain

1% Methylene blue (100 ml)

Ingredients	Composition
Methylene blue chloride	1 gm
Ethanol	10 ml
Distilled water, to make	100 ml

Place the required amount of the methylene blue into the volumetric flask containing the require amount of the ethanol. Shake well the contents until completely dissolved and add the distilled water to make the final volume upto100 ml.

Appendix-III

3. Decontaminating agents and culture media preparation

A. Solutions for Perroff's method

1. NaOH solution

Stock solution: 40 % NaOH (10 M) (100 ml)

Ingredients	Composition
NaOH pellet	40 gm
Distilled water 100ml	

Working solution: 4 % NaOH (500 ml)

Ingredients	Composition
40 % NaOH	50 ml
Distilled water	450 ml

2. Solutions for Nekal-BX

Ingredients	Composition
Nekal	0.1g
NaOH	5g
Na3PO4	1.0g
Distilled Water	1000ml

CaCl2 + BaCl2 solution

Ingredients	Composition
CaCl2	2g
BaCl2	4g
Distilled water	100ml

B. Culture media

1. Modified Ogawa media (MOG)

i) Mineral salt solution (1000ml)

Ingredients
Composition

Potassium dihydrogen phosphate anhydrous (KH2PO4) 20.0g

Magnesium citrate, quadrihydrate (Mg3 (C6H5O7) 2.4H2O) 1.0g

Sodium glutamate	5.0g
Distilled water 1000ml	

Dissolve the ingredients in distilled water by heating. Autoclave it at 121°C for 30 minutes to sterilize it. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

ii) Malachite green solution, 2%

Ingredients Composition	
Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

iii) Homogenised whole eggs

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and homogenize them in a sterile mixer grinder.

iv) Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Ingredients Composition	

Mineral salt solution	1000ml
Malachite green solution	40 ml
Whole eggs homogenate	2000ml
Glycerol	40ml

The resulting pH of the medium is 6.8. The medium is mixed well and distributed in 6-8ml volumes in sterile 20ml volumes in 20x150mm screw-capped test tubes.

v) Coagulation of medium

Place the tubes in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C.

vi) Sterility check

After inspissation, the whole media batch or a representative sample of culture tubes should be incubated at 37°C for 24 hours as a check of sterility.

vii) Storage

The medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out.

2. Modified Lowenstein Jensen media (MLJ)

i) Mineral salt solution (1000ml)

Ingredients Composition	
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	4.0g
Magnesium sulphate ($MgSO_4$)	0.4g
Magnesium citrate, quadrihydrate ($Mg_3(C_6H_5O_7)_2.4H_2O$)	1.0g
Asparagine	6.0g
Sodium glutamate	5.0g
Distilled water 1000ml	

Dissolve the ingredients in distilled water by heating. Autoclave it at 121°C for 30 minutes to sterilize it. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

ii) Malachite green solution, 2%

Ingredients Composition	
Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

iii) Homogenised whole eggs

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and homogenize them in a sterile mixer grinder.

iv) Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Ingredients Composition	
Mineral salt solution	1000ml

Malachite green solution	33 ml
Whole eggs homogenate	1610ml
Glycerol	20ml

The resulting pH of the medium is 6.8. The medium is mixed well and distributed in 6-8ml volumes in sterile 20ml volumes in 20x150mm screw-capped test tubes.

v) Coagulation of medium

Place the tubes in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C.

vi) Sterility check

After inspissation, the whole media batch or a representative sample of culture tubes should be incubated at 37°C for 24 hours as a check of sterility.

vii) Storage

The medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out.

3. 2% Buffered Lowenstein Jensen media (BLJ)

i) Mineral salt solution (1000ml)

Ingredients Composition	
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	20.0g
Magnesium sulphate ($MgSO_4$)	0.4g
Magnesium citrate, quadrihydrate ($Mg_3(C_6H_5O_7)_2.4H_2O$)	1.0g
Asparagine	6.0g
Sodium glutamate	5.0g
Distilled water 1000ml	

2% Buffered Lowenstein Jensen media is prepared by addition of 16g of Potassium dihydrogen phosphate anhydrous (KH_2PO_4) into the commercially available Modified Lowenstein Jensen media to increase its concentration from 0.4% to 2%. Dissolve the ingredients in distilled water by heating. Autoclave it at 121°C for 30 minutes to sterilize it. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

ii) Malachite green solution, 2%

Ingredients Composition	
Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

iii) Homogenised whole eggs

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and homogenize them in a sterile mixer grinder.

iv) Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Ingredients Composition	
Mineral salt solution	1000ml
Malachite green solution	33 ml
Whole eggs homogenate	1610ml
Glycerol	20ml

The resulting pH of the medium is 6.8. The medium is mixed well and distributed in 6-8ml volumes in sterile 20ml volumes in 20x150mm screw-capped test tubes.

v) Coagulation of medium

Place the tubes in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C.

vi) Sterility check

After inspissation, the whole media batch or a representative sample of culture tubes should be incubated at 37°C for 24 hours as a check of sterility.

vii) Storage

The medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out.

Appendix-IV

4. Biochemical tests

A. Nitrate Reduction Test

1. Reagents

Sodium nitrate substrate in buffer

Prepare 0.01M sodium nitrate in 0.022M phosphate buffer, pH 7.0 as follows:

Ingredients	Composition
KH2PO4	3.02g
Distilled water	1000ml

Dissolve potassium phosphate in distilled water
to provide an 0.022M solution .....Solution 1

Ingredients	Composition
Na2HPO4	3.16g
Distilled water	1000ml

Dissolve sodium phosphate in distilled water
to provide an 0.022M solution .....Solution 2

Add 611ml of solution 2 to 389ml of solution 1 , and mix well.
Check pH to be 7.0 .....Solution 3

Complete sodium nitrate substrate buffer

Ingredients	Composition
NaNO 3	0.85g
Solution 3	1000ml

Dissolve the sodium nitrate in the buffer and dispense in 100ml aliquots. Sterilise by autoclaving at 121°C for 15 minutes. When needed, aliquots of the substrate solution are aseptically dispensed into sterile screw-capped tubes in 2ml quantities.

Hydrochloric acid solution

Ingredients	Composition
Concentrated HCI	10ml
Distilled water	10ml

Slowly add concentrated HCI to distilled water (never the reverse) to obtain a 1:1 dilution. Store the reagent in an amber bottle in the dark in the refrigerator.

Sulfanilamide solution, 0.2%

Ingredients	Composition
Sulfanilamide	0.2g
Distilled water	100ml

Dissolve sulfanilamide in distilled water and store in an amber bottle in the dark in a refrigerator.

N-naphthylethylene-diamine solution, 0.1%

Ingredients	Composition
N-naphthylethylene-diamine	0.1g
Distilled water	100ml

Dissolve naphthylethylene-diamine in distilled water and store in an amber bottle in the dark in a refrigerator.

2. Controls

Control the reagents by testing the extract from an uninoculated tube of medium (negative control) and use an extract from a culture of *M. tuberculosis* H37Rv as positive control.

3. Procedure

- Add 0.2ml of sterile saline to a screw-cap tube
- Use a sterile loop/spade to emulsify two loopfuls/spadefuls of 4-week old culture in the saline

- Add 2ml of the NaNo3 substrate buffer solution into the tubes
- Shake well and incubate upright in a 37°C water bath for 3 hours
- Transfer the supernatant solution to a serilized test tube
- Add in the following order: 1 drop diluted HCI (shake tube well), 2 drops 0.2% sulfanilamide and 2 drops 01.% N-napthtylethylene-diamine
- Examine immediately for a pink to red colour and compare to colour standard

4. Results and interpretation

Negative: No colour. If no colour develops, the test is either negative or the reduction has proceeded beyond nitrite. Add a small amount of powdered zinc to all negative tests by tipping the end of a slightly moistened applicator stick into dry zinc and shaking into the liquid. If nitrate is still present, it will be catalysed by the zinc and a red colour will develop, indicating a true negative. If no color develops the original reaction was positive but the nitrate was reduced beyond nitrite. Repeat the test to confirm the observation

Positive: Red color which vary from pink to very deep red-crimson:

Faint pink = +/-Clear
pink = 1+
Deep pink = 2+
Red = 3+
Deep red = 4+
Purplish red = 5+
Only 3+ to 5+ is considered positive.

B. Nitrate test with paper strips

Paper test strips for the detection of nitrate following nitrate reduction are commercially available. The paper strip test method yields most consistent results with mycobacteria that vigorously reduce nitrate, such as M. tuberculosis. It therefore provides reliable results and is much less labor-intensive than the chemical method, but is much more

expensive. In this experiment we used combined Nitrate Niacin test so the chemicals used in this case are also same as in Nitrate reduction test.

1. Procedure

- Add 0.2ml of sterile saline to a screw-cap tube
- Use a sterile loop/spade to emulsify two loopfuls/spadefuls of 4-week old culture in the saline
- Add 2ml of the NaNo3 substrate buffer solution into the tubes
- Shake well and incubate upright in a 37°C water bath for 3 hours and remove
- Transfer the supernatant solution to a serilized test tube
- Use sterilised forceps and carefully insert a nitrate test strip (arrow indicates which end to insert first); do not let the strip contact any fluid on the side of the tube

2. Results and interpretation

Negative: No color

Positive: Yellow liquid in the bottom of the tube. Discard any colour on the stip itself; this may occur because of oxidation of chemicals, especially at the top of the strip.

C. Catalase

1. Reagents

0.067M phosphate buffer solution, pH 7.0

Ingredients	Composition
Na2HPO4 anhydrous	9.47g
Distilled water	1000ml

Dissolve disodium phosphate in distilled water
to provide an 0.067M solution .....Solution 1

Ingredients	Composition
KH2PO4	9.07g
Distilled water	1000ml

Dissolve monopotassium phosphate in distilled water to provide an 0.067M solution .....Solution 2

Hydrogen peroxide, 30%

30% hydrogen peroxide (H2O2), also known as Superoxol (Merck) is stored in the refrigerator.

Tween 80, 10%

Ingredients	Composition
Tween 80	10ml
Distilled water	90ml

Mix Tween 80 with distilled water and autoclave at 121°C for 10 minutes. The Tween may settle during autoclaving and may be resuspended by swirling immediately after autoclaving and during cooling. Store in the refrigerator.

Complete catalase reagent (Tween-peroxide mixture)

Immediately before use, mix equal parts of 10% Tween 80 and 30% hydrogen peroxide. Allow 0.5ml reagent for each strain to be tested.

2. Controls

Drop method

Use an uninoculated tube of as negative control and an LJ butt of *M. tuberculosis* H37Rv as positive control Heat liable test (68ºC, pH 7.0) for identification of *M. tuberculosis.*

3. Procedure

- With a sterile pipette, aseptically 0.5 ml of 0.067/M phosphate buffer, pH 7.0 to screw caped tubes.
- Several loopfulls of test cultures was suspended in the buffer solution using sterile loops.
- The tubers containing the emulsified cultures were placed in a previously heated water bath at 68ºC for 20 minutes. Time and temperature was critical.
- The tubes were removed from the heat and allowed to cool to room temperature.
- 0.5ml of freshly prepared Tween-peroxide was added to each tube and caps were replaced loosely.
- 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.

4. Result and interpretation

Positive : Bubbles

Negative : No bubbles

D. Growth on medium containing p-Nitrobenzoioc Acid (PNB)

In laboratories where facilities and reagents for niacin and nitrate testing are not available, identification of tubercle bacilli may be done by a combination of one or more of the catalase tests described previously together with growth at 25°C on LJ medium and growth on LJ medium containing p-nitrobenzoic acid at 37°C. Problems with incubation at 25°C 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.

1. Procedure

- Inoculate two slopes of LJ medium containing glycerol and one tube of LJ medium containing p-nitrobenzoic acid (PNB) at a concentration of 500mg/litre

- Incubate one LJ slope and the PNB slope at 37°C in an internally illuminated incubator and examine at 3, 7, 14 and 21 days. When growth is evident on the LJ slope examine it for pigment. If an internally illuminated incubator is not available, remove slopes from the dark incubator as soon as growth is evident, loosen the caps to admit some oxygen and expose them to daylight (but not direct sunlight) or place 1m from a laboratory bench lamp for 1 hour. Reincubate and examine for pigment the following day.

- Incubate the other LJ slope at 25°C and examine at 3, 7, 14 and 21 days

2. Results and interpretation

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

Appendix-V

5. Grading of results

A. Grading of sputum smear microscopy results for ZN staining

Smear examination were reported as per the guidelines given by IUATLD

Finding	Report
No AFB per 300 oil immersion fields	Negative
1-9 AFB per 100 oil immersion fields	Record exact number
10-99 AFB per 100 oil immersion fields	1+
1-10 AFB per oil immersion field	2+
> 100 AFB per oil immersion field	3+

B. Grading of sputum smear microscopy results for Auramine staining

Smear examination were reported as per the guidelines given by IUATLD

Finding	Report
No AFB	Negative
1-10 AFB per slide	Doubtful
< 1 AFB per field / 10 AFB per slide	1+
1-9 AFB per fields	2+
10-100 AFB per field	3+
> 100 AFB per field	4+

B. Grading of culture results

Culture results were reported as per guidelines given by IUATLD, the cultures were observed till 9 weeks before discarding it as negative

Finding	Report
Contamination	Contamination
No growth	Negative
Less than 20colonies	Report exact number
20-100 colonies	1+
100-200 colonies	2+
More than 200 colonies but not confluent growth	3+
Confluent growth	4+

Appendix-VI

6. Statistical analysis of the results

Table I Comparison between MLJ and MOG

	Positive	Negative	Contamination	Total
MLJ	371	39	30	440
MOG	405	24	11	440
Total	776	63	41	880

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
371	388	17	289	0.74
405	388	17	289	0.74
39	31.5	7.5	56.25	1.79
24	31.5	7.5	56.25	1.79
30	20.5	9.5	90.25	4.40
11	20.5	9.5	90.25	4.40

H0: Σ Oi = Σ Ei There no significant difference between MLJ and MOG according growth and contamination rates.

H1: Σ Oi $\neq$ Σ Ei There is significant difference between MLJ and MOG according growth and contamination rates.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 13.866$

Thus $\chi 2$ cal (13.866) > $\chi 2$ tab at $\alpha = 0.01$ and df = (3-1) x (2-1) = 2 i.e. 13.82

Hence, H0 is rejected. There is significant difference between MLJ and MOG according growth and contamination rates at 99% level of significance.

Table II Comparison between MLJ and BLJ

	Positive	Negative	Contamination	Total
MLJ	371	39	30	440
BLJ	407	19	14	440
Total	778	58	44	880

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
371	389	18	324	0.83
407	389	18	324	0.83
39	29	10	100	3.45
19	29	10	100	3.45
30	22	8	64	2.91
14	22	8	64	2.91

H0: Σ Oi = Σ Ei There no significant difference between MLJ and MOG according growth and contamination rates.

H1: Σ Oi $\neq$ Σ Ei There is significant difference between MLJ and MOG according growth and contamination rates.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 14.38$

Thus $\chi 2$ cal (14.38) > $\chi 2$ tab at $\alpha = 0.01$ and df = (3-1) x (2-1) = 2 i.e. 13.82

Hence, H0 is rejected. There is significant difference between MLJ and BLJ according growth and contamination rates at 99% level of significance.

Table III Comparison between MOG and BLJ

	Positive	Negative	Contamination	Total
MOG	405	24	11	440
BLJ	407	19	14	440
Total	812	43	25	880

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
405	406	1	1	0.00
407	406	1	1	0.00
24	21.5	2.5	6.25	0.29
19	21.5	2.5	6.25	0.29
11	12.5	1.5	2.25	0.18
14	12.5	1.5	2.25	0.18

H0: Σ Oi = Σ Ei There no significant difference between MOG and BLJ according growth and contamination rates.

H1: Σ Oi $\neq$ Σ Ei There is significant difference between MOG and BLJ according growth and contamination rates.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 0.95$

Thus $\chi 2$ cal (0.95) < $\chi 2$ tab at $\alpha = 0.05$ and df = (3-1) x (2-1) = 2 i.e. 5.99

Hence, H0 is accepted. There is no significant difference between MOG and BLJ according growth and contamination rates.

Table IV Comparison of Decontamination techniques based on result of MLJ

	Positive	Negative	Contamination	Total
Nekal	192	12	16	220
NaOH	179	27	14	220
Total	371	39	30	440

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
192	185.5	6.50	42.25	0.23
179	185.5	-6.50	42.25	0.23
12	19.5	-7.50	56.25	2.88
27	19.5	7.50	56.25	2.88
16	15	1.00	1.00	0.07
14	15	-1.00	1.00	0.07

H0: Σ Oi = Σ Ei There no significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MLJ.

H1: Σ Oi $\neq$ Σ Ei There significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MLJ.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 6.36$

Thus $\chi 2$ cal (6.36) > $\chi 2$ tab at $\alpha = 0.05$ and df = (3-1) x (2-1) = 2 i.e. 5.99

Hence, H0 is rejected. There significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MLJ.

Table V Comparison of Decontamination techniques based on result of MOG

	Positive	Negative	Contamination	Total
Nekal	204	10	6	220
NaOH	201	14	5	220
Total	405	24	11	440

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
204	202.5	1.5	2.25	0.01
201	202.5	1.5	2.25	0.01
10	12	2	4	0.33
14	12	2	4	0.33
6	5.5	0.5	0.25	0.05
5	5.5	0.5	0.25	0.05

H0: Σ Oi = Σ Ei There no significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MLJ.

H1: Σ Oi $\neq$ Σ Ei There significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MLJ.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 0.25$

Thus $\chi 2$ cal (0.25) < $\chi 2$ tab at $\alpha = 0.05$ and df = (3-1) x (2-1) = 2 i.e. 5.99

Hence, H0 is accepted. There no significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on MOG.

Table VI Comparison of Decontamination techniques based on result of BLJ

	Positive	Negative	Contamination	Total
Nekal	204	7	9	220
NaOH	203	7	10	220
Total	407	14	19	440

Observed Value (Oi)	Expected Value (Ei)	(Oi - Ei)	(Oi - Ei)2	(Oi - Ei)2/ Ei
204	203.5	0.5	0.25	0.00
203	203.5	0.5	0.25	0.00
7	7	0	0	0.00
7	7	0	0	0.00
9	9.5	0.5	0.25	0.03
10	9.5	0.5	0.25	0.03

H0: Σ Oi = Σ Ei There no significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on BLJ.

H1: Σ Oi $\neq$ Σ Ei There significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on BLJ.

Test statistics is $\chi 2$

From $\chi 2 = \Sigma (Oi - Ei)2/ Ei$ $\chi 2 = 0.6$

Thus $\chi 2$ cal (0.6) < $\chi 2$ tab at $\alpha = 0.05$ and df = (3-1) x (2-1) = 2 i.e. 5.99

Hence, H0 is accepted. There no significant difference between Perroff's method and Nekal-BX method according growth and contamination rates shown on BLJ.

Appendix-VII

Master Chart of Culture Results

(is in the excel file)

Appendix-VIII

Results of Biochemical Tests

S.N.	Lab. No.	Growth on PNB containing Media	Niacin-Strip test	Nitrate Reduction test	Catalase test at 27°C	Catalase test at 68°C
1	2199C	No Growth	Positive	Positive	Positive	Negative
2	2243A	No Growth	Positive	Positive	Positive	Negative
3	2212C	No Growth	Positive	Positive	Positive	Negative
4	2235B	No Growth	Positive	Positive	Positive	Negative
5	2251F	No Growth	Positive	Positive	Positive	Negative
6	2297A	No Growth	Positive	Positive	Positive	Negative
7	2234B	No Growth	Positive	Positive	Positive	Negative
8	2264A	No Growth	Positive	Positive	Positive	Negative
9	2298F1	No Growth	Positive	Positive	Positive	Negative
10	2282A	No Growth	Positive	Positive	Positive	Negative
11	2297B	No Growth	Positive	Positive	Positive	Negative
12	2318A	No Growth	Positive	Positive	Positive	Negative
13	2365A	No Growth	Positive	Positive	Positive	Negative
14	2365C	No Growth	Positive	Positive	Positive	Negative
15	2340E	No Growth	Positive	Positive	Positive	Negative
16	2390C	No Growth	Positive	Positive	Positive	Negative
17	2419B	No Growth	Positive	Positive	Positive	Negative
18	2457B	No Growth	Positive	Positive	Positive	Negative
19	M274	No Growth	Positive	Positive	Positive	Negative
20	2499B	No Growth	Positive	Positive	Positive	Negative
21	2508B	No Growth	Positive	Positive	Positive	Negative
22	2545B	No Growth	Positive	Positive	Positive	Negative
23	2557A	No Growth	Positive	Positive	Positive	Negative
24	2557B	No Growth	Positive	Positive	Positive	Negative
25	2593A	No Growth	Positive	Positive	Positive	Negative
26	2669A	No Growth	Positive	Positive	Positive	Negative
27	2674F	No Growth	Positive	Positive	Positive	Negative
28	2704B	No Growth	Positive	Positive	Positive	Negative
29	2722B	No Growth	Positive	Positive	Positive	Negative
30	2805F	No Growth	Positive	Positive	Positive	Negative
31	2825A	No Growth	Positive	Positive	Positive	Negative
32	2856A	No Growth	Positive	Positive	Positive	Negative
33	2861B	No Growth	Positive	Positive	Positive	Negative
34	2877C	No Growth	Positive	Positive	Positive	Negative
35	2959A	No Growth	Positive	Positive	Positive	Negative

36	2943C	No Growth	Positive	Positive	Positive	Negative
37	3011A	No Growth	Positive	Positive	Positive	Negative
38	3049A	No Growth	Positive	Positive	Positive	Negative
39	3085A	No Growth	Positive	Positive	Positive	Negative
40	3115A	No Growth	Positive	Positive	Positive	Negative
41	3169A	No Growth	Positive	Positive	Positive	Negative
42	3183B	No Growth	Positive	Positive	Positive	Negative
43	3282C	No Growth	Positive	Positive	Positive	Negative
44	3217A	No Growth	Positive	Positive	Positive	Negative