CHAPTER I

1. INTRODUCTION

Tuberculosis (TB) is a disease of great antiquity and has almost century caused more suffering and death than any other infection. TB is a well known air borne disease caused by bacterium *Mycobacterium tuberculosis*. Based on the site of involvement of infection and host response, TB is of different types; pulmonary tuberculosis, extra pulmonary tuberculosis and disseminated tuberculosis (Cole et al., 2005). Despite the availability of effective chemotherapy, it continues to be the major public health problem in the most countries of the world.

According to WHO approximately one-third of the world's population is affected with TB bacilli.1 in 10 people infected with TB bacilli will lead to active TB in their life time. If not treated, each person with active TB infects on average 10 to 15 people per year. There were 9.4 million new TB cases in 2008. Sub-Saharan, Africa and Southeast Asia bear the highest number of cases. The vast majority of deaths are in the developing world, with more than half of deaths occurring in Asia (WHO, 2009).

In Nepal, TB is one of the major public health problem. It is estimated that about 45% of the total population is infected with TB, out of which 60% are in the productive age group. Every year, 44,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. Introduction of treatment by DOTS has already reduced the number of the deaths; however 8,000-11,000 people continue to die every year from this disease (NTP 2006/07).

Classical methods for the diagnosis of mycobacteria species rely on staining specimens for acid–fast bacilli followed by culture, colony and cell morphology, growth rate and subsequent biochemical testing. Acid fast microscopy is the fastest, easiest and least expensive tool for the rapid identification of patients with mycobacterial infections but the method is unable to distinguish within the mycobacterium genus (Salfinger, 1994). Microscopy has a sensitivity and specificity low enough to be useful as a presumptive screening test. Although relatively insensitive, microscopy remains the only same day diagnostic test within the budget of

all laboratories as acid fast results are rapidly available. Culture technique is considered as the gold standard for the confirmatory diagnosis of presence of mycobacteria, as it has high degree of sensitivity and specificity, however it is labor intensive and time consuming. Tuberculin skin test was originally a test for active disease but it is unsuitable as it has limited specificity. The radiographic appearance for the diagnosis of pulmonary mycobacteriosis is unreliable because other chest diseases resemble TB on X-ray (WHO, 2000).

The conventional methods used for the diagnosis of mycobacterial pulmonary diseases are clinical symptoms, chest X-ray, sputum smear microscopy, tuberculin skin test; vitro culture methods have their own limitations. To overcome all these limitations, we need to have novel molecular technique LAMP that combines the rapidity of microscopy and the sensitivity of culture to identify the mycobacterium species would be great help to the clinician during the initial treatment of the patient.

Although of lesser public health importance than *M. tuberculosis*, Non Tuberculosis Mycobacterium (NTM) were widely recognized as human pathogens only in 1950s, when NTM were isolated from several large series of patients with NTM lung disease were reported (Horsburgh et al., 1996). The routine diagnosis method for tuberculosis cannot diagnose these NTM. It requires either biochemical test or molecular techniques. These NTM are collectively known as atypical, anonymous, tuberculoid, opportunist and mycobacterium other than tuberculosis bacilli (MOTT). Although there are more than a dozen species of atypical mycobacterium, the most common are *Mycobacterium kansasii, Mycobacterium avium* and *Mycobacterium intracellular.* Although the *M. avium* and *M. intracellular* are distinct species and can be identified and distinguished by DNA probes (Accuprobe, gene probe etc). They are almost indistinguishable phenotypically. So they are called as *M. avium intracellular* complex (MAIC, MAC or MAI) (Forbes et al., 2000).

Identification of the species of mycobacterial isolates is another critical requirement for clinical laboratories because most first line antituberculosis drugs have less invitro activity against *M. avium* complex and *M. kansasii*. Usually, identification of Mycobacteria in most of the hospitals and research laboratories in our country do not reach up to the species level. All suspected tuberculosis cases, when showed growth

on culture media, referred to as tuberculosis and physician start treatment of tuberculosis. These practiced activities are the main burden for the effective control and management of tuberculosis. So, proper use of effective drugs is only the key requirement for the treatment of tuberculosis caused by atypical mycobacterium.

The LAMP-based assay can identify *M. tuberculosis* complex, *M. avium, M. intracellular* and *M. kansasii* from sputum samples directly within 1 hour. In LAMP the reagents reacts under isothermal conditions with high specificity, efficiency and rapidity but a separate test must be performed for each species. Hence the LAMP assay is more advantageous than all of the currently available DNA probe methods in its simple operation and rapid reaction. Loop-Mediated Isothermal Amplification (LAMP) is a method introduced by Notomi et al. (2000) which has characteristics that may allow its use in less sophisticated settings. This method employs DNA polymerase and a set of six specially designed primers that recognize a total of eight distinct sequences on the target DNA using strand displacement reaction which is completed at constant temperature (at 64^0 C). It provides high amplification efficiency with DNA being amplified 10^9-10^{10} times within 15-60min. It produces extremely large amounts of amplified products which are stem-looped DNA with several inverted repeats of the target and cauliflower like structure with multiple loops and can be identified by the simple detection method such as visual judgment by the turbidity or fluorescence of the reaction mixture (Eiken, 2005).

This study had been aimed to evaluate the use of LAMP for the rapid and direct detection of Mycobacterial species including *M. tuberculosis* complex, *M. avium, M. intracellulare* and *M. kansasii* directly from sputum specimens in Nepal. The results obtained by this new method were evaluated by comparing it to culture and microscopy. LAMP is totally new technique in our country and this study will help to establish the LAMP in hospital laboratories for rapid diagnosis of tuberculosis as well as other atypical Mycobacteria for effective treatment and case management of TB in our country.

CHAPTER II

2. OBJECTIVES

2.1 General objective

To standarize Loop-Mediated Isothermal Amplification (LAMP) for rapid detection of pulmonary disease due to *M. tuberculosis* complex, *M. avium, M. intracellulare* and *M. kansasii* in sputum specimens in Nepal.

2.2 Specific objectives

1. To screen and identify *M. tuberculosis, M. avium, M. intracellulare, and M. kansasii* rapidly in the sputum samples using LAMP.

2. To compare sensitivity and specificity of LAMP with culture and sputum smear microscopy techniques for the diagnosis of tuberculosis in Nepal.

3. To analyse socio-demographic profile and disease characteristics of culture positive patients.

CHAPTER III

3. LITERATURE REVIEW

3.1 Pulmonary tuberculosis

Tuberculosis is a leading cause of death in the world from a bacterial infectious disease caused by the *M. tuberculosis* .The disease primarily affects lungs called as pulmonary TB and can also infect intestine, meninges, bones, joints, lymph glands, skin and other tissues of the body called as extra-pulmonary tuberculosis (Park, 2000).

Despite the availability of effective chemotherapy, the disease affects 1.8 billion people every year which is equal to the one-third of the entire world population. HIV positive people infected by *M. tuberculosis* have 20 folds higher chance of developing TB than normal people. Even in HIV positive cases tuberculosis can be cured if diagnosed in time and treated properly (WHO, 2010). The emergence of MDR-TB and XDR-TB and the co-infection of TB with HIV / AIDS patients have been considered as a global burden of these diseases. WHO declared global emergency in 1993 and the stop TB partnership proposed a global plan to stop tuberculosis which aims to save 14 million lives between 2006 and 2015 and eliminate TB as public health problem in 2050 (WHO, 2007).

Compared with tubercle bacilli, NTM are of low virulence and, although man is frequently infected, overt disease is very uncommon except in those who are profoundly immunosuppressed. The opportunist mycobacterial diseases are localized lymphadenitis, tuberculosis-like pulmonary lesions, skin lesions following traumatic inoculation of bacteria, disseminated disease (Greenwood, 2002).

M. avium complex (MAC), followed by *M. kansasii*, are the most frequent pathogens causing lung disease. *M. avium* is the isolate in more than 95% of patients with AIDS who develop MAC infections. *M. intracellulare* is responsible for 40% of such infections by nontuberculous mycobacteria in patients with AIDS. The prevalence of *M. kansasii* has increased with the HIV pandemic other atypical pathogens occasionally causing pulmonary disease include *M. abscessus, M. scrofulaceum, M. marinum, M. fortuitum, M. szulgai, M. simiae, M. xenopi, M. malmoense, M. celatum, M. asiatium and M. shimodii* (Falkinham et al., 2002).

3.2 Mycobacteria

Organisms belonging to the genus mycobacterium are very thin, pleomorphic, straight or slightly curved rods (1-4 x 0.2-0.5 µm) and non motile (Cheesbrough, 1989). They are aerobic, non capsulated and acid and alcohol fast organisms. The genus belongs to the mycobacteraceae family, Actinomycetales order and Actinomcetes class (Forbes et al., 2000). The high G+C content of the DNA of Mycobacterium species (61 to 70 mol% except for M. leprae, 55%) is within the range of those of the other mycolic acid containing genera including Nocardia (64 to72 mol %), Rhodococcus (63 to73 mol %), Gordonia (63to 69 mol %) and Tsukamurella (68 to 74 mol %) (Good fellow, 1998).

Mycobacterium species have a characteristic cell wall structure containing N-glycolymuramic acid instead of N-acetylmuramic acid than other bacteria, which is rich in lipid content. The hydrophobic property is due to the possession of thick, complex, lipid rich and waxy cell walls. A further important characteristic is acid-fastness, or resistance to decolorization by a dilute mineral acid (or alcohol) after staining with hot carbol fuchsin or other arylmethane dyes.

The name 'Mycobacterium' meaning fungus like bacterium is derived from mould like appearance of *M. tuberculosis* when growing in liquid media. The genus contains over 80 named species of mycobacteria including human and animal pathogens are normally saprophytes (Greenwood, 2002). The pathogen includes the organism that causes human and bovine tuberculosis and leprosy. Clinically, mycobacteria are divided into two major groups: those associated with tuberculosis are collectively known as M. *tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum)* and others associated with opportunist diseases are collectively known as non-tuberculous mycobacteria (NTM). The latter group has been several collective names such as atypical, anonymos, tuberculoid and Mycobacteria other than tuberculosis bacilli (MOTT) (Forbes et al., 2000).

3.2.1 *Mycobacterium tuberculosis*

M. tuberculosis is non-sporing, non capsulated, straight and slightly curved rod measuring 1-4×0.2-0.5µm (Cheesbrough, 1989**)**. In sputum and other clinical specimens, they may occur singly or in clumps, and in sputum and other liquid culture, they often grow as twisted rope like colonies termed serpentine cords (Greenwood, 2002). Tubercle

bacilli are aerobes, grow slowly with generation time 12-24 hours, colonies usually appears in 2-3 weeks and may sometimes requires eight weeks incubation, optimum temperature 37°C and pH 6.4-7.0. If gram stain is performed, *M. tuberculosis* either stain very weakly gram positive, or not at all. The tubercle bacilli grow on Lowenstein-Jensen (LJ) medium is widely used. The colonies of *M. tuberculosis* on LJ medium are dry, rough, and creamy or buff colored (Cheesbrough, 1989**)**.

The cell wall structure of *M. tuberculosis* is the major determinants of virulence for the bacterium. Over 60% of the mycobacterium cell wall is lipid which consists of three major components, mycolic acid, cord factor and wax-D. Mycolic acids are strong hydrophobic molecules which prevent the attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. Cord factor is responsible for the serpentine cording which is toxic to mammalian cells and is also inhibitor of PMN migration. Wax-D in the cell envelope is the major component of Freund's complete adjuvant (Forbes et al., 2000).

3.2.2 *Mycobacterium avium* complex and *Mycobacterium kansasii*

M. avium complex are slow growing, non-sporing, non-capsulated and indistinguishable morphologically from those of *M. tuberculosis*. They are referred to as non-photochromogenic as they do not produce pigment colonies on incubation either in light or dark. Typical culture of *M. avium* complex (MAC) on L-J medium is smooth, yellow colored and they are non-adherent (they do not produce cord factor) (Forbes et al., 2000).

M. kansasii is a non-sporing, non-capsulated, straight rod. Morphologically, it is similar to that of *M. tuberculosis*. It belongs to a group of Mycobacteria referred to as photochromogenic because when grown in light or when young colonies are exposed briefly to light, colonies become brilliant yellow but when colonies grown in dark are non pigmented. Prolonged light exposure may induce production of dark red beta-carotene crystals on the surface and inside of colony The colonies of *M. kansasii* on L-J medium are smooth, yellow colored and they are non-adherent (they do not produce cord factor) (Forbes et al., 2000).

3.3 Transmission

All new infection with tuberculosis is acquired by air borne transmission. Main source of infections are droplet infection or droplet nuclei containing viable virulent organism generated by patients with pulmonary tuberculosis during coughing, sneezing and vocalizing. Single cough can produce 3,000 - 5,000 droplet nuclei (Park, 2005). The incubation period generally ranges from 3 to 6 weeks and sometimes up to months or years (Park, 2000).

There is no evidence of animal-to-human or human–to-human transmission of pulmonary infection due to *M. avium*, *M. intracellular*, *M. kansasii*, *M. fortuitum* and other NTM are rare (Falkinham et al., 2002). Human disease is suspected to be acquired from environmental NTM. Airborne NTM may play an important role for the source of infection in respiratory disease (Tanaka et al., 2000). MAC are common in many environmental sites, including water and soil. Aerosols of fresh and salt water may contain MAC and is transmitted via inhalation through the respiratory tract and ingestion via the GI tract (Horsburgh et al., 1996).

3.4 Pathogenesis

TB infection means that MTB is in the body, but the immune system is keeping the bacteria under control. The immune system does this by producing macrophages that surround the tubercle bacilli .The cells form a hard shell that keeps the bacilli contained and under control. Most people with TB infection have a positive reaction to tuberculin skin test. People who have TB infection but not TB disease are not infectious, i.e., they cannot spread the infection to other people. These people usually have a normal chest x-ray. TB infection is not considered a case of TB disease (WHO, 2004).

Only 3-4% of infected individuals will develop active disease upon initial infection, 5-10% within 1 year. These percentages are much higher if the individual is HIV positive (EDCD, 2003).

Early events:-Tuberculosis begins when droplet nuclei containing bacilli reach the alveoli, where they multiply virtually unrestricted within inactivated macrophages until the macrophages burst. Other macrophages also phagocytes MTB, but they are also inactivated and hence, cannot destroy bacteria. Then the lymphocytes begin to infiltrate.

The lymphocyte, especially T-cells recognize processed and presented MTB antigen in context of MHC molecules which result in T-cell activation and liberation of cytokines including gamma interferon (IFN). IFN causes in the activation of macrophages which are capable of destroying MTB. It is at this stage that the individual becomes tuberculin-positive which is the result of the host developing a vigorous cell mediated immune (CMI) response. Although CMI response is necessary to control an MTB infection, it is also responsible for much of the pathology associated with tuberculosis (Catran et al., 2000). Activated macrophages and T-cells also secrete cytokines that play a role in the development of immune pathology, including interleukin1, tumor necrosis factor (TNF), and gamma IFN (Stenger et al., 1997). The tubercle formation begins, in which MTB cannot multiply because of low pH and anoxic environment, however, MTB can persists within these tubercles for extended periods (Cantran et al., 2000)

Later events:-Although many activated macrophages can be found surrounding the tubercles tuberculosis are postulated to be unable to multiply due to its acidic pH, low availability of oxygen, toxic fatty acids, and some bacilli may remain dormant but alive for decades. The strength of the host cellular immune response determines whether an infection is arrested here or progresses to the next stages. This enclosed infection is referred to as latent or persistent TB and can persist throughout a person's life in an asymptomatic and non transmissible state. In person with efficient cell mediated immunity, the infection may arrest permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions (Kaufmann et al., 2002). However, if an infected person cannot control the initial infection in the lung or if a lastly infected person's immune system is weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by an unknown process and then serves as a rich medium in which the now revived bacteria can replicate in an uncontrolled manner. At this point, viable MTB can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (milliary or extra pulmonary TB). When this happens, the person becomes infectious and requires antibiotic therapy to survive (Issar et al., 2003).

Infections caused by *Mycobacterium avium* are common in AIDS patients and patients with chronic lung diseases. The bacterium can be acquired both through the intestinal route and respiratory route. *M. avium* is capable of invading mucosal epithelial cells and translocating across the mucosa. *M .avium* is capable of invading type II alveolar epithelial cells and can replicate within these cells. The bacterium can infect macrophages, interfering with several functions of the host cell. The host defense against *M. avium* is primarily dependent on CD4+ T lymphocytes and natural killer cells. Activated macrophages can inhibit or kill intracellular bacteria by mechanisms that are currently unknown, but *M. avium* can invade resting macrophages and suppress key aspects of their function by triggering the release of transforming growth factor beta and interleukin 10. Co-infection with HIV-1 appears to be mutually beneficial, with both organisms growing faster (Bermudez et al., 2000).

Lipoarabinomannans (LAMs) are glycolipids from *Mycobacterium kansasii* stimulated mRNA expression and secretion of TNF-α and IL-8 from human macrophage-like differentiated THP-1 cells (Somoskovi et al., 2000).

M. kansasii infections of the lung are likely acquired by inhalation of the organism. Once inside the lung, the bacteria attach to CR3 (CD 11b/ CD18) on resident alveolar macrophages and are internalized into phagosomal compartments that do not fuse with lysosomes on produce super oxide. Lipoarabinomannans (LAMs) are glycolipids from *Mycobacterium kansasii* stimulated mRNA expression and secretion of TNF-α and IL-8 from human macrophage-like differentiated THP-1 cells (Cecile et al., 2003). The macrophage provides an ideal environment for the bacterium to multiply within and carries the organism to other tissues where it eventually form the characteristic thin-walled cavitary lesion associated with this disease (Le cabec et al., 2000).

3.5 Risk factors

An individual's risk of infection depends on the extent of exposure to droplet nuclei and susceptibility to the infection .The risk of infection of a susceptible individual is high with close, prolonged indoor exposure to a person with sputum smear positive PTB. The risk of transmission of infection from a person with sputum smear negative PTB is low and with extra pulmonary TB is even lower (WHO, 2005).

The risk of becoming infected is largely exogenous in nature, determined by the characteristics of the source case, environmental and duration of exposure. The risk of developing tuberculosis given that infection has occurred is largely endogenous, determined by integrity of the cellular immune system (Rieder, 1995). These factors includes HIV infection, diabetes mellitus, chronic lung diseases, cancer, advanced kidney disease, malnutrition, disease for which steroid therapy is prescribed, alcoholism, heavy smoking, the elderly, and uses of intravenous drugs (WHO, 2010).

In patients infected with HIV, disseminated NTM infections typically occurred only after CD4 T-lymphocyte number had fallen below 50/µl, suggesting that specific T-cell products or activities are required for mycobacterial resistance (Horsburgh et al., 1996).

In the HIV-uninfected patient group, genetic syndromes of disseminated NTM infection have been associated with specific mutations in the interferon (IFN)- γ and the interleukin (IL) 12 synthesis and the response pathways (Casanova et al., 2002).

There is also an increased risk of NTM infection where there is an association between bronchiectasis with nodular pulmonary NTM infections and a particular body habitus, predominantly in postmenopausal women (e.g. pectus excavatum, scoliosis, mitral valve prolapsed) (Buschman et al., 1991).

3.6 Treatment and control

The Standard short course treatment of tuberculosis therapy consists of isoniazid, rifampicin, pyranizamide and ethambutol for 2 months, then isoniazid and rifampicin alone for a further 4 months. The patients are considered cured at six months (although there is a still relapse rate of 2 to 3%). For latent TB, standard treatment is 6-9 months of isoniazid alone (WHO, Treatment of TB Guidelines for National Programmes).

Most first-line anti tuberculosis drugs have 10-100 times less in vitro activity against *M. avium* complex isolates than against *M. tuberculosis*. This diminished activity may be due to the lipophilic cell wall of *M. avium* complex, which prevents drug penetration (Rastogi et al., 1981). Treatment *of M. avium complex* pulmonary disease include a regimen of daily clarithromycin (500 mg twice a day) or azithromycin (250 mg), rifampin (600 mg) or rifabutin (300 mg), and ethambutol (25 mg/kg for 2 months, then 15 mg/kg) is recommended for therapy of adults not infected with the HIV virus. Streptomycin two to

three times per week should be considered for the first 8 wk as tolerated. Patients should be treated until culture-negative on therapy for 1 year. Treatment therapy of disseminated *M. avium* complex disease in adults should include daily clarithromycin (500 mg twice a day) or azithromycin (250 to 500 mg), plus ethambutol 15 mg/kg per day. Consideration should be given to the addition of a third drug (preferably rifabutin at a dose of 300 mg/d). Therapy should be continued for life until more data becomes available (Wallace et al., 1997).

Treatment of *M. kansasii* pulmonary disease include a regimen of daily isoniazid (300 mg), rifampin (600 mg), and ethambutol (25 mg/kg for 2 month, then 15 mg/kg) for 18 month with a minimum of 12 month culture negativity is recommended for pulmonary disease in adults caused by *M. Kansasii* (Clarithromycin or rifabutin will need to be substituted for rifampin in HIV-positive patients who take protease inhibitors (Wallace et al., 1997).

3.7 Diagnosis

Diagnosis of active disease includes clinical suspicion, chest radiographs, staining for acid-fast bacilli, and culture for mycobacterium, more recently, nucleic acid amplification assay (Foulds and O'Brien, 1998).

3.7.1 Clinical Diagnosis

Clinical signs and symptoms develop in only a small proportion (5-10 percentages) of infected healthy people (McMurray, 2001). Clinically, pulmonary tuberculosis is chiefly present with persistent cough for 3 or more weeks, haemoptysis, shortness of breath and chest pain, loss of appetite and loss of weight, malaise, fatigue, night sweats and fever Symptoms of extra pulmonary tuberculosis depend on the organ involved (Enarson et al., 2000).

Chronic pulmonary disease is the most common clinical manifestation of NTM (Falkinham, 1996; Wolinsky, 1979). Signs and symptoms MAC-PD and MK-PD are variable and non-specific. However, virtually all patients have chronic or recurring cough. Other symptoms of variably include sputum production, fatigue, malaise, dyspnea, fever, hemoptysis and weight loss can also occur, usually with advanced disease. Evaluation is often complicated by the symptoms caused by co-existing lung

diseases. These conditions include chronic obstructive airway diseases associated with smoking, bronchiectasis, previous mycobacterial diseases, cystic fibrosis and pneumoconiosis (Iseman et al., 1991).

3.7.2 Radiographic diagnosis

The radiographic appearance can also help in the detection of pulmonary mycobacteriosis but they do not allow etiological diagnosis. Radiographic presentation of tuberculosis includes upper-lobe infiltrates, cavitary infiltrates and hilar or paratracheal adenopathy. There is a radiological difference between primary and secondary tuberculosis. Primary disease is usually characterized by single lesion in the middle or lower right lobe with enlargement of the draining lymph nodes. The X-ray presentation of pulmonary *Mycobacterium avium* complex (MAC) in the immunocompetent can be variable. The classic radiographic appearance of pulmonary MAC is indistinguishable from that of pulmonary tuberculosis (Levin, 2002). Another type of clinical presentations of MAC-PD frequently observed by X-Ray is parenchymal disease and cavitary diseases. The parenchymal infection (progenitive focus) appears in the apical or posterior portion of the upper lobes. Thoracic cage abnormalities are also appear in some MAC-PD patients by the radiographic findings (Levin et al., 2002). Chest radiograph of MAC-PD patients with HIV / AIDS has alveolar or interstitial infiltrates that are either diffuse or are not focal to the upper lobes (Modilevsky et al., 1989).

The chest radiograph of pulmonary *M. kansasii* infection is similar to that of pulmonary tuberculosis. Approximately 90% of patients with disease caused by *M. kansasii* have cavitary infiltrates (Ahn et al., 1982).

3.7.3 Tuberculin skin test

Infection in an asymptomatic individual can be diagnosed with the help of the intradermal PPD skin test. Intradermal introduction of PPD into a previously infected hypersensitive person results in the delayed (42-72 hours) appearance of an indurated (raised, hard) reaction with or without erythema. It is impossible to distinguish between present past infection based on a positive tuberculin test (McMurray, 2001). The tuberculin skin test also 5 fails to detect a substantial proportion of persons co-infected

with HIV and of person with advanced tuberculosis. Its sensitivity for active disease varies considerably from 65% to 94% (Lalvani et al., 2001).

3.7.4 Laboratory Diagnosis

3.7.4.1 Smear Microscopy

Acid fast microscopy is the most commonly used technique in the routine diagnosis of mycobacteria. Two types of acid-fast stains are used to detect mycobacteria: Ziehl-Neelsen carbolfuchsin stain or Kinyoun carbolfuchsin stain, and the auramine O or auramine-rhodamine fluorochrome stain. In the carbol fushin (Ziehl-Neelsen), acid-fast organism appear red against a blue background, while in the fluorochrome staining, the acid fast organisms appear as fluorescent rods, yellow to orange (the color may vary with the filter system used) against a pale or orange background. Of the carbolfuchsin stains, the Kinyoun stain is technically the easier to use; however, the performance of this stain was reported to be inferior to that of the Ziehl-Neelsen stain in a survey of 75 field clinics in Bangladesh (Van Deun et al., 2005), as well as in U.S. laboratories participating in a New York state proficiency program (Somoskovi et al., 2001). Despite these observations, the fluorochrome stain is currently recommended for use in U.S. laboratories. Steingart et al. (2006) reviewed 45 relevant studies and concluded that fluorescent microscopy was more sensitive than and as specific as carbolfuchsin stains.

Whenever disease is suspected, three specimens must be collected for examination by microscopy which increases the predictive value of positivity of smear microscopy, reaching almost that of culture. Whenever possible, they should be obtained within twenty-four hours (WHO, 2000). The overnight specimen is more likely to be positive than the spot specimens. The cumulative positivity is 31%, 93% and 100% for first, second and third sputum respectively (NTP, 2002).

Although the specificity of acid-fast microscopy is excellent (i.e., 100%), the sensitivity is not optimal, and this method is unable to distinguish within the mycobacterium germs. The sensitivity of microscopy is influenced by numerous factors such as the prevalence and severity of tuberculosis or NTM disease. The type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing, the method of centrifugation, and most importantly by the staining technique

and the quality of examination (Kent and Kubica, 1985). Therefore, this method detects 5,000-10,000 bacteria per ml with sensitivity range between 46-78% (Kox, 1996).

It is recommended that a negative result should be reported only following the examination of at least 300 microscopic oil-immersion view fields. Recently, in order to increase the efficacy of acid-fast microscopy, a model of a computer-directed automated microscope was constructed. The examination of smears by this technique could well increase the sensitivity (Somoskovi et al., 1999).

3.7.4.2 Culture:

Isolation of mycobacteria by culture is a highly sensitive diagnostic method, requiring only 10 to 100 viable bacilli per ml. of cultured material for detection of bacilli. The sensitivity of culture is excellent, ranging from 80% to 93% and specificity is quite high, at 98% (ATS-CDC, 2000). Moreover, culture is more sensitive for the detection of mycobacteria than acid-fast microscopy as well as to determine colony morphology, drug susceptibility tests and genotyping of particular cultured NTM. There are two types of culture media: solid media, which include egg-based media (Lowenstein-Jensen and Ogawa medium) and agar based media (Middle brook 7H10 and 7H11]. Cultures should be inoculated onto one or more solid media and into a liquid medium. At least three respiratory (sputum) cultures should be used for the initial evaluation. The two tubes of LJ media are inspected weekly for mycobacterial growth for 8 weeks.

M. tuberculosis is an obligate aerobe, grows optimally at 37°C (range: 25°C to 40°C) and pH 6.4 -7.0. It is a slow growing organism with generation time of 14 -15 hours. On solid medium *M. tuberculosis* forms a dry, rough, raised, irregular colony with wrinkled surface. The colonies are creamy white initially, becoming yellowish or buff colored later and tough when picked off. They are tenacious and not easily emulsified. Until, recently, identification of *M. tuberculosis* from positive cultures depend on biochemical tests for niacin, aryl sulphatase, neutral red, catalase-peroxidase, amidase, and nitrate reductase after the incubation of 2 to 3 weeks (Forbes et al., 2000).

Wallenstein Media are recommended for use in the cultivation and isolation of mycobacteria other than tuberculosis. It is comprised of an egg yolk medium supplemented with glycerol and malachite green and particularly useful in the recovery of

Mycobacteria avium complex (Forbes et al., 2000). The media is examined within five to seven days, and weekly thereafter for up to eight weeks. Each type of colony morphology, pigment, and growth rate and biochemical testing is required for definitive identification.

The time to detection of growth of a mycobacterial species can be shortened greatly by the use of an automated or semi-automated liquid culture system that can detect growth much earlier than the naked eye i.e., obtaining results by one or two weeks over solid. For drug susceptibility testing (DST), the interval may be reduced to as little as ten days, compared with 28 to 42 days with conventional solid media. Liquid systems are more sensitive for detection of mycobacteria and may increase the case yield by 10% over solid media. The first such system to be used was BACTEC 460TB. Since then new systems that rely on non radiometric detection of growth have been developed, such as the MB/Bac T (Organon Tenika), BACTEC 9000 (Becton Dickinson) and the mycobacterial growth indicator tube (BD BACTEC MGIT 960), BD BACTEC MGIT 320 system. With increased sensitivity and reduced delays, liquid systems may contribute significantly to improved patient management. But, however for maximum recovery of Mycobacteria, a combination of both M960 and LJ media should be used as 1.93% isolates could be detected by LJ only (S. Rishi et al, 2007).

3.8 Other diagnostic techniques for pulmonary Mycobacteriosis

3.8.1 Immunological diagnostic methods

a) Antigen –detection

Mycobacterial antigens have been detected by enzyme-linked immunosorbent assay (ELISA) in sputum and cerebrospinal fluid and by latex agglutination assay in cerebrospinal fluid. The assay could be used for the diagnosis of any mycobacterial disease, including disease caused by the *Mycobacterium avium-Mycobacterium intracellulare* complex (Lenlka et al., 2000).

b) Enzyme linked immunospot assay for detection of Interferon- γ

Recent studies demonstrated that an enzyme-linked immunospot (ELISPOT) assay for interferon-γ (IFN-γ) produced by activated T cells after exposure to antigens of *M. tuberculosis*, early secretory antigenic target 6 (ESAT-6), and culture filtrate protein 10

(CFP-10) is a specific method for identifying *M. tuberculosis* infection (Hill et al., 2004).

c) Antibody Detection

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

3.8.2 Molecular Techniques for the Diagnosis of Pulmonary Mycobacteriosis

A wide range of molecular techniques have been developed which offer many advantages over conventional methods of identification. The results are obtained rapidly, are reliable and reproducible. Molecular techniques have shown their usefulness for the identification of most mycobacterium. Probes are widely used in clinical laboratories for the identification of the most common mycobacterial species. Because automated DNA sequencing and the programs for analyzing sequence data have become technically simpler, polymerase chain reaction-based sequencing is now used in many mycobacterial reference laboratories as a routine method for species identification (Chemlal et al., 2003)

They include nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR), transcription-mediated amplification (TMA), oligonucleotide ligation amplification and strand displacement amplification (SDA). Each of these amplification methods has its own innovation to re-initiate new rounds of DNA products to promote the next round of DNA synthesis. 3SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify the target sequence. Similarly, SDA eliminates the heat denaturation step in cycling DNA synthesis by employing a set of restriction enzyme digestions and strand displacement DNA synthesis with modified nucleotides as substrate (Walker et al., 1992).

a) Nucleic acid hybridization methods

Use of the Acuprobe (Gen-probe Inc, San Diego, CA) nucleic acid hybridization kits represented a quantum leap forward in the rapid identification of the MTB complex, the *M. avium* complex (MAC), *M. gordonae* and *M. kansasii*. These assays allowed rapid identification of these mycobacteria (Metchock et al., 1999).

b) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is still the most popular amplification method that allows the production of more than 10 million copies of a target DNA sequence from only a few molecules.PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated round of denaturation, primer annealing and primer extension (Mullis and faloona, 1987). PCR based assay can also be used for the detection of *MTB* complex, *M. avium*, *M. intracellular* and *M. kansasii* by using species-specific primer.

c) Strand Displacement Amplification (SDA)

SDA is an isothermal amplification process that takes advantages of ability of DNA polymerase to start at the site of a single-stranded nick in double-stranded DNA, extend one strand from the 3 end, and displace the downstream strand of DNA (Walker et al, 1992). The replicated DNA and the displaced strands are then substrates for additional round of oligonucleotide annealing, nicking, and strand displacement such that the amplification proceeds in a geometric manner and can produce 10^7 to 10^8 fold amplification in about 2 hours (Bloom, 1994). Species-specific SDA assays have been developed for *M. tuberculosis, M. avium, and M. kansaii*.

d) Transcription-Mediated Amplification (TMA)

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

3.9 Loop-mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target

DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single- stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA structure. The cycling reaction continues with accumulation of 10^9copies of target in less than an hour. Amplification and detection of gene can be completed in single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (at 64^0c). The final products are stem- loop DNAs with several inverted repeats of the target and cauliflower- like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity (Iwamoto et al., 2003).

LAMP reaction appears to be limited only by amount of deoxyribonucleoside triphosphate and primers. LAMP sensitivity is not significantly influenced by the presence of non-target DNA (Notomi et al., 2000). In the process, a large amount of pyrophosphate ion is produced, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by-product. This phenomenon allows easy and rapid visual identification that the target DNA was amplified by LAMP (Mori et al., 2001). The resulting amplicons are visualized also by adding fluorescence dye to the reaction tube or with gel electrophoresis in the same way as for PCR (Iwamoto et al., 2003). Therefore, LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of any infectious disease both in well-equipped laboratories and in field situations.

Furthermore the addition of two more primers called loop primers shortens reaction time for the amplification by one third to one half. Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primers (Nagamine et al., 2002).

3.9.1 Characteristics of LAMP

LAMP has the following characteristics: (i) all reaction can be conducted under isothermal conditions ranging from 60^0 C to 65^0C by using only one type of enzyme: (ii) the specificity of the reaction is extremely high because it uses four primer recognizing six distinct regions on the target DNA; (iii) amplification can be performed in a shorter time than amplification by PCR because there is no time loss due to thermal cycling; (iv) it produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube (Iwamoto et al., 2003); (v) the total cost can be reduced, as LAMP does not require special reagents or sophisticated equipments; (vi) the amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand, and (vii) LAMP amplifies target RNA at an isothermal temperature using thermo-stable reverse transcriptase (RT LAMP). In conclusion, LAMP serves as a very useful method for DNA diagnosis (Eiken, 2005).

3.9.2 The principle of LAMP method (Eiken, 2005)

Basic principle

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed (Figure.1).

STEP1

As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA.

STEP2

Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP.

STEP3

The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.

STEP4

A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.

STEP5

The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions.

STEP6

The single strand DNA in Step (5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.

STEP7

Double stranded DNA is produced through the processes described in Step (6).

STEP8

The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method (LAMP cycling). The above process can be understood as producing the starting structure for LAMP cycling.

Basic principle 8-11 (Cycling Amplification step)

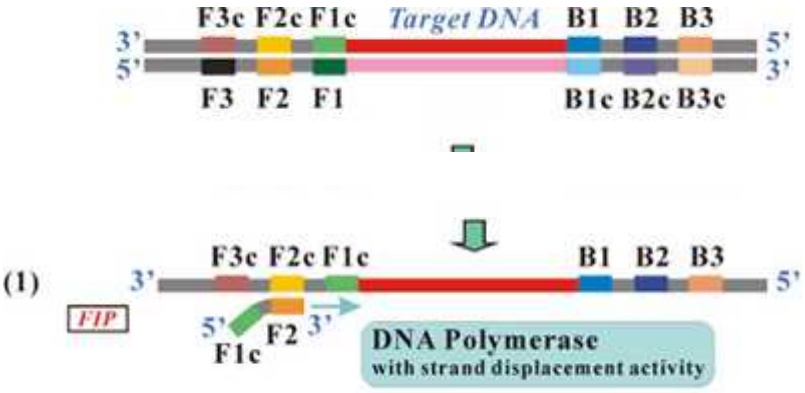
A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA

and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

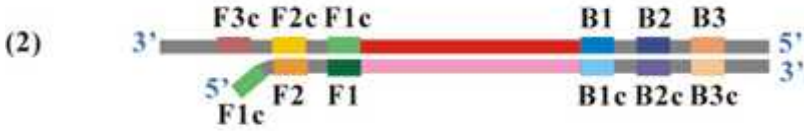
Principle of Loop Primers (Eiken, 2005)

The loop primers (either loop Primer B or loop Primer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure provide and increased number of starting points for DNA synthesis for the LAMP method. There is an amplified product containing six loops. In the original LAMP method, four of these loops would not be used, but through the use of loop Primers, all the single stranded loops can be used as starting points for DNA synthesis.

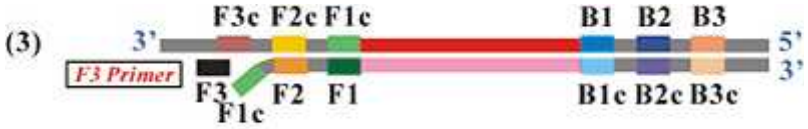
STEP I



STEP 2



STEP3



STEP4

STEP5

STEP6

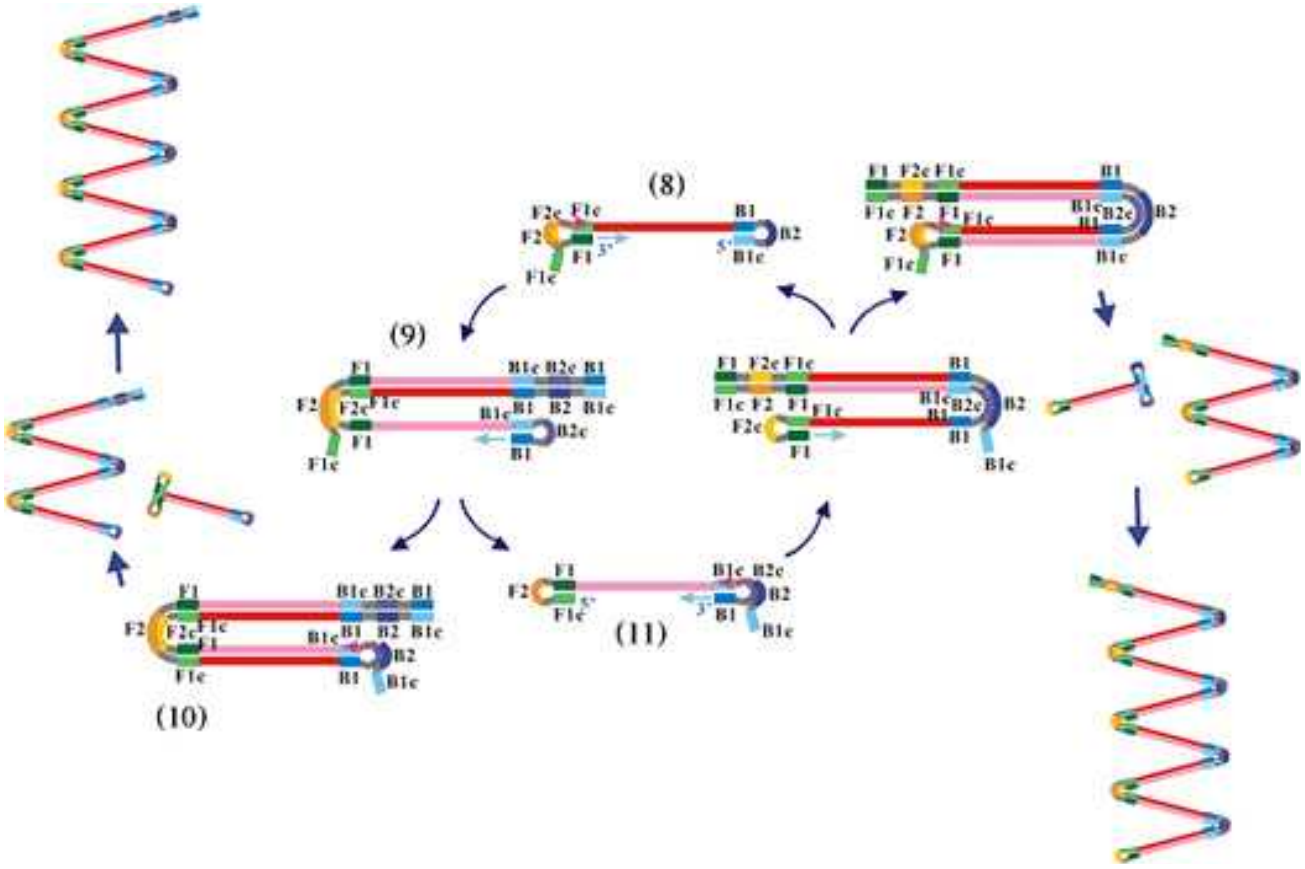
STEP7

(7)

STEP8

(8)

STEP8-11 (Cycling Amplification step)



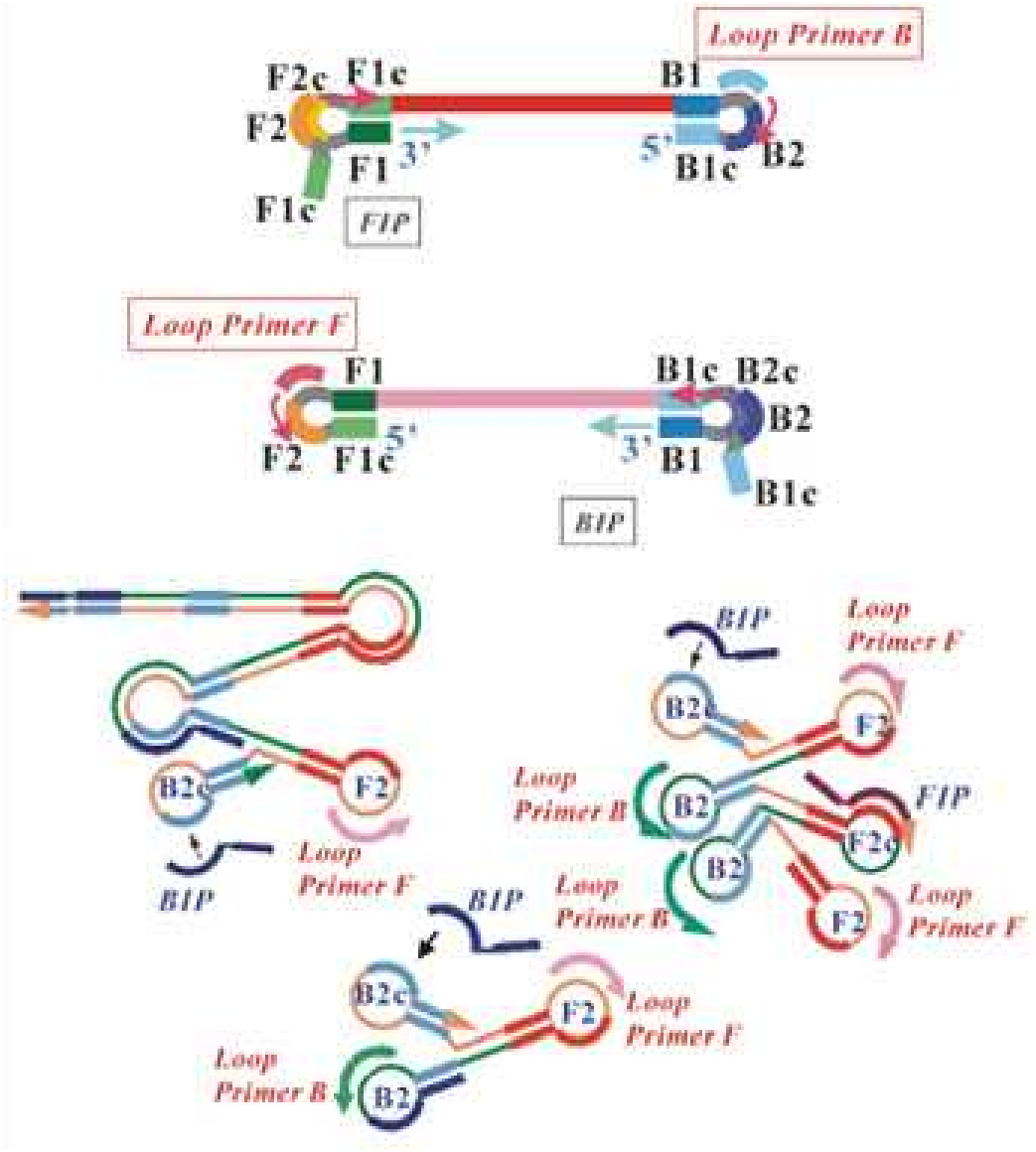


Figure.1 Mechanism of loop-mediated isothermal amplification (Eiken chemical Co. Ltd.)

3.9.3 Components of LAMP

LAMP Buffer

LAMP buffer containing Tris-HCl (pH 8.8), KCl, NH_4SO_4, and 0.1% Triton X-100 keeps the reaction at a proper pH so that the LAMP reaction takes place.

$MgSO_4$

Magnesium sulphate is an important component of LAMP. Since magnesium concentration is a crucial factor affecting the performance of *Bst* polymerase, addition of $MgSO_4$ in the reaction mixture of LAMP provided free magnesium.

Betaine (N, N, N-trimethylglycine)

The presence of betaine stimulates the rate of reaction and increase target selectivity with a significant reduction in amplification of irrelevant sequences (Notomi et al., 2000). It has been suggested that betaine affects the extension reaction either by binding to AT pairs in the major groove or by increasing the hydration of GC pairs by binding within the minor groove and thus destabilizing GC-rich DNA (Mytelka et al., 1996)

Deoxyribonucleoside triphosphate (dNTPs)

The dexyribonucleoside triphoshpates (dATP, dCTP, dTTP, dGTP) provide both the energy and nucleoside for synthesis of DNA. It is important to add equal amount of each nucleotide to the LAMP mixture to prevent mismatches of bases.

***Bst* DNA polymerase**

Bst DNA polymerase large fragment is the portion of the *Bacillus stearothermophilus* DNA polymerase protein that contains the 5 -3 polymerase activity, but lacks the 5-3 exonuclease activity. It has the applications for DNA sequencing through high GC regions and rapid sequencing from nanogram amounts of DNA template (New England BioLabs, Inc. 2003). It adds the deoxynucleotide to the DNA template and retains the auto-cycling strand displacement DNA synthesis in the LAMP.

Primer

It is a short segment of nucleotides, which is complementary to a section of the DNA, which is to be amplified. LAMP employs a set of four specially designed primers that recognize a total of six distinct sequences on the garget DNA. The use of Loop primers shortens this reaction time of the amplification.

Template DNA

It is a piece of DNA present in sample, which will be amplified by the LAMP. Successful amplified LAMP employs a set of four specially designed primers with two Loop primers that recognize a total of eight distinct sequences on the target DNA.

Fluorescent Detection Reagent

Calcein in Fluorescent Detection Reagent as initially combined with manganese ions to achieve the quenching effect. The amplification generates the by- product, pyrophosphate ions, which will bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. From this feature, the presence of fluorescence can indicate the presence of target gene and visual detection can be achieved.

3.9.4 Procedure of LAMP

The procedure of LAMP simply consists of preparation of samples (target gene i.e., DNA or RNA), mixing of LAMP reagents (primers, DNA polymerase, dNTPs, reaction buffer, and reverse transcriptase in case of RNA), and incubating whole mixture at 64^0C for 1 hours to allow detection of amplified products (Eiken, 2005)

As mentioned above, LAMP method allows the whole reaction process, including denaturing, proceeds at a constant temperature by incubating the reagents in a simple incubator. Without the electrophoresis, the presence of amplified product can be detected in a short time directly with naked eye observing the white turbidity of magnesium pyrophosphate, a byproduct of the LAMP reaction (Mori et al., 2001). LAMP amplicons in the reaction tube are also directly detected with naked eye by adding fluorescence dye to the tube and observing the color of the solution. The solution turns green in the presence of a LAMP amplicon, while it remains orange with no amplification (Iwamoto et al., 2003).

3.9.5 Sensitivity of LAMP

LAMP is highly sensitive and able to detect DNA at as few as six copies in the reaction mixture. It requires a strict condition for the recognition of six distinct sequences in the

target DNA. It is less prone to the presence of irrelevant DNA than PCR (Notomi et al., 2000).

3.9.6 Primers for *M. tuberculosis, M. intracellular, M. avium* and *M. kansasii.*

Primers used in the amplification process can be either species-specific or genus- specific (Kent et al., 1995).

Genus-specific Primers

A clinical specimen may contain *M. tuberculosis* and other mycobacteria. Therefore there are advantages to use genus specific primers in the amplification. The amplified product could then be identified with species-specific probes. Laboratories have used combination of Mycobacterium-species primers for amplification, and then amplified product has been hybridized with specific probes or analyzed by RFLP. Sometimes restriction fragment profiles of known organisms were normalized to a fixed distance and the similarities of patterns were calculated by using a computer-aided comparison program. Patterns of unknown organisms could then be compared with this database for identification (Kent et al., 1995).

Species-specific Primers

These primers determine the specificity of the reaction. Yamamoto and Haryama (1995) designed a set of primers that allowed both the amplification of the *gyrB* gene, which encodes the submit B protein of DNA gyrase (topoisomerase II). The rate of molecular evolution inferred from *gyr B* gene sequences is faster than that inferred from 16SrRNA gene sequences.

For detection of *M. tuberculosis*, *M. avium*, *M. intracellular* and *M. kansasii* and other Mycobacterial species, primers specific to that of *gyr B* gene have developed for LAMP.

3.9.7 Primers for LAMP (Eiken, 2005)

Design of 4 types of primers (described in detail below) based on the following 6 distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side and B1, B2 and B3 regions at the 5' side.

FIP: Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the5' end.

F3 primer: Forward Outer Primer consists of the F3 region that is complementary to the F3c region.

BIP: Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.

B3 Primer: Backward Outer Primer consists of the B3 region that is complementary to the B3c region.

Main Points of Primer Design (Eiken, 2005)

Proper primer design is crucial for performing LAMP amplification. The above primer regions can be determined by using the Primer Explore (special software to design LAMP primers) after considering the base composition, GC contents and the formation of secondary structures. T_m value can be obtained by Nearest Neighbor method. The following is the main points of primer design:

1. Distance between Primer regions

- The distance between 5' end of F2 and B2 is considered to be 120-180 bp, and the distance between F2 and F3 as well as B2 and B3 is 0-20bp.
- The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) is 40-60 bp.

2. T_m value for Primer region

- About 60^0 C - 65^0 C in the case of GC rich and normal, about 55^0 C - 60^0 C for AT rich.

3. The stability of Primer end.

- The dG calculated on 6bp from the following end regions should be less than 4kcal/mol, 5' end of F1c/B1c and 3' end of F2/B2 as well as F3/B3.

4. GC contents

- About 50-60% in the case of GC rich and normal, about 40-50% for AT rich.

5. Secondary Structure

- Primers should be designed so as not to easily form secondary structures. 3' end sequence should not be AT rich or complementary to other primers.

6. Others

- If the restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products.

3.9.8 Gyrase B gene (*gyr*B)

PCR, which permits the amplification of specific DNA sequences and multiplies even a single copy of a given DNA sequence by a factor of 1012, has been applied to various fields of diagnosis and has proved to be most useful tools for the rapid diagnosis of infectious diseases (Gaydos et al., 2002). PCR has been used to analyze various mycobacerial genes for diagnostic purposes, including 16S and 23S rRNA genes, genus- and species-specific fragments in the chromosome (De Wit et al., 1990), genes coding for the 65-Da heat shock protein and the 38-kDa protein B antigen, the dnaJ gene, and insertion sequences such as IS 6110 (Eisenach et al., 1990). 16S rRNA has been reported to be a suitable target for use in PCR amplification assays for the detection of *Mycobacterium spp*. in a variety of clinical samples and has frequently been used to identify various specific microorganisms because 16S rRNA genes show species-specific polymorphisms (Holberg et al., 1999). However, because of the extremely show speed of the molecular evolution of 16S rRNA, the number of substituted bases between the 16S rRNA genes of closely related bacterial strains, such as those belonging to the *M. tuberculosis* complex, is either nonexistent or too small to differentiate between these species.

As an alternative to 16S rRNA anlaysis, Yamamoto and Harayama,1995 designed a set of PCR primers that allowed both the amplification of the *gyrB* gene, which encodes the subunit B protein of DNA gyrase (topoisomerase type II), and the rapid nucleotide sequencing of the amplified gyrB fragments from a wide variety of bacteria. They used these gyrB genes in the taxonomic classification of *Pseudomonas putida* and *Acinetobacter* strains. The rate of molecular evolution inferred from *gyr*B gene sequences is faster than that inferred from 16S rRNA gene sequences. For detection of Mycobacterium species, Kasai et al. have determined the *gyrB* gene sequences of 43

slowly growing strains belonging to 15 species in the genus Mycobacterium and developed a method of PCR and PCR-restriction fragment length polymorphism analysis to differentiate these species (Kasai et al., 2000).

3.9.9 LAMP for Diagnosis of TB, MAC-PD, MK-PD and other Diseases

Iwamoto et al. (2003) used LAMP for detection of *M. tuberculosis* complex, *M. avium* and *M. intracellular* directly from sputum specimens as well as for detection of culture isolates grown in a liquid medium or on a solid medium (Ogawa's medium). Species-specific primers were designed by targeting the *gyr*B gene, and their specificities were validated on 24 mycobacterial species and 7 nonmycobacterial species. The whole procedure was quite simple, starting with the mixing of all reagents in a single tube, followed by an isothermal reaction during which the reaction mixture was held at 64^0C. The resulting amplicons were visualized by adding SYBR Green 1 to the reaction tube. The assay had a detection limit of 5 to 50 copies of purified DNA with a 60-min incubation time. The reaction time could be shortened to 35 min for the species identification of *M. tuberculosis* complex, *M. avium* and *M. intracellulare* from a solid medium culture. Residual DNA lysates prepared for the amplicor assay from 66 sputum specimens were tested in the LAMP assay. Although the sample size used for the latter assay was small, 2.75 « l of the DNA lysates, it showed a performance comparable with that of the Amplicor assay, which required 50 « l of the lysates or for a sputum specimen that contained a corresponding amount of DNA available for testing.

Enosawa et al. (2003) evaluated the usefulness of LAMP in detecting specific gene sequences of cultured *M. avium* subsp. paratuberculosis (MAP). A total of 102 primer sets for LAMP was designed to amplify the IS900, HspX, and F57 gene sequences of MAP. Using each of two primer sets (P-1 and P-2) derived from the IS900 fragment; it was possible to detect MAP in a manner similar to that used with nested PCR. The sensitivity of LAMP with P-1 was 0.5pg/tube, which was more sensitive than nested PCR. When P-2 was used, 5pg/tube could be detected, which was the same level of sensitivity as that for nested PCR. LAMP with P-1 was specific. Although only 2 *M. scrofulaceum* strains out of 43 non-MAP mycobacterial strains were amplified, the amplification reaction for these strains was less efficient than for MAP strains, and their

products could be distinguished from MAP products by restriction digestion, LAMP with P-2 resulted in very specific amplification only from MAP, the same result obtained with nested PCR. These results indicate that LAMP can provide a rapid yet simple test for the detection of MAP.

Hara-Kudo et al. (2005) used LAMP assay to detect Salmonella within 60 min. The 220 strains of 39 serotypes of *Salmonella* subsp. enterica and 7 strains of *S. enterica* subsp. arizonae were amplified, but not 62 strains of 23 bacterial species other than *Salmonella*. The sensitivity of the LAMP assay was found to be > 2.2 cfu/test tube using nine serotypes. The specificity was similar to that of a PCR assay, but the sensitivity of LAMP was greater. Both fluorescence and turbidity were able to detect the products in the LAMP assay. *S. enteritidis* in a liquid egg sample artificially inoculated with the organism was detected by the LAMP assay at 2.8 cfu/test, although negative by PCR assay.

Song et al. (2005) reported LAMP method for detecting *Shigella* and *Escherichia coli*. The target for this LAMP method was the *ipa*H gene which was carried by both of the pathogens. The LAMP method efficiently detected the gene within 2 hours at a minimal amount of bacteria (8CFU) per reaction. Kuboki et al. (2003) reported conditions for a highly sensitive, specific, and easy diagnostic assay based on LAMP technology for the detection of parasites in the *Trypanosoma brucei* group (including *T. brucei brucei*, *T. brucei gambiense, T. brucei rhodesiense, T. evansi* and *T. congolense*). They showed that the sensitivity of the LAMP-based method for detection of trypanosomes in vitro was up to 100 times higher than that of PCR-based method. In vivo studies in mice infected with human-infective *T. brucei gambiense* further highlighted the potential clinical importance of LAMP as a diagnostic tool for the identification of African typanosomiasis.

Saito et al. (2005) developed and evaluated LAMP assay for the rapid detection of *Mycoplasma pneumoniae*. The assay specifically amplified only *M. pneumoniae* sequences, and no cross-reactivity was observed for other *Mycoplasma species* or rspiratory bacterial species. The detection limit for this assay was found to be 2×10^2 copies, corresponding to 2-20 color changing units of *M. pneumoniae* in 1 hour, as observed in a real-time turbidimeter and electrophoretic analysis. The accuracy of the LAMP reaction was confirmed by restriction endonuclease analysis as well as direct

sequencing of the amplified product. The assay was applied to 95-nasopharyngeal swab samples collected from patients or from healthy individuals, and compared to a real-time PCR assay in house. A concordance of 100% was observed between the two assays.

Annaka (2003) designed and evaluated a new DNA amplification method in order to detect *Legionellae.* The primer targeting 16S rRNA gene we designed in order to detect a wide range of *Legionella* species. They could specifically detect *Legionella* species including *L. pneumophila, L. anisa, L. bozemanii, L. dumoffii, L. erythra, L. feeleii, L. gormanii, L. logbeachae, L. micadadei, L. oakridgensis*, and *L. sainthelensi*. The detection limit of the assay was 6 cfu per test of *L. pneumpohilia* strain. Furthermore, all of the positive LAMP results could be obtained within 50 minutes. The LAMP method was able to detect a wide range of *Legionella* species with high specificity, sensitivity, rapidity, and a simple procedure. Ushikubo (2004) mentioned that LAMP method had shown prominent results of surpassing the performance of the conventional gene amplification method. They believed LAMP technology with the integration of many features, can rightly apply to clinical genetic testing, food and environmental analysis, as well as nucleic acid test in different fields.

Maruyama et al. (2003) used in Situ LAMP to detect stxA (2) gene in *Escherichia coli* O157:H7 cells. The mild permeabilization conditions and low isothermal temperature used in the in situ LAMP method caused less cell damage than in situ PCR. It allowed use of fluorescent antibody labeling the bacterial mixture after the DNA amplification for identification of *E. coli* O157:H7 cells with a stxA (2) gene. Higher-contrast images were obtained with this method than with in situ PCR.

To evaluate the usefulness of LAMP for diagnosing central nervous system infection with herpes simplex virus (HSV), Kimura et al. (2005) compared the LAMP method with real-time PCR, using samples that were previously tested by nested PCR. They examined 69 cerebrospinal fluid (CSF) samples from patients suspected of having HSV infection of the central nervous system. When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 10%, the positive predictive value was 100%, and the negative predictive value was 90%.

Leo et al. (2006) used LAMP for detection of *Plasmodium falciparum* DNA directly on heat–treated blood sample of suspected patients. To evaluate the LAMP, blood-film microscopic analyses, PCR assay were used as comparision method which showed sensitivity and specificity of LAMP were 95% and 99% respectively. Unlike PCR, LAMP assay doesn't require purified DNA for efficient DNA amplification, thereby reducing the cost and turnaround time for *P.falciparum* diagnosis.

Hidekazu et al. (2009) applied LAMP to detect *Leishmania donovani* D.N.A in blood samples. This technique detected 1fg of D.N.A which was 10-fold more sensitive than a conventional PCR. All nested PCR positive blood samples were positive with LAMP.

3.10 Reverse Transcription- Loop-Mediated Isothermal Amplification (RT-LAMP)

Fujino et al. (2005) developed a new sensitive and rapid method to detect the measles virus genome by reverse transcription loop-mediated isothermal amplification (RT-LAMP). They examined 50 nasopharyngeal secretion (NPS) samples that were obtained during the 1999 outbreak and stored at -70^0 C and fresh NPS, lymphocytes and sera from 11 patients in 2003. Total RNA was extracted from the samples and subjected to reverse transcription-polymerase chain reaction (RT-PCR) and RT-LAMP. They detected genomic RNA corresponding to at least 0.01-0.04 TCID50, 30-100 copies in samples by RT-LAMP within 60 min after extraction of RNA, and all four genotypes isolated in Japan were equally amplified. Specific DNA amplification was monitored spectrophotometrically by real time turbidimeter and the quantity of RNA was calculated. Measles virus genome was detected in 44 of 50 stored NPS by RT-PCR and in 49 by RT-LAMP. The vaccine strain was discriminated from wild strains after sequencing the LAMP products.

Emily et al. (2009) used reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assay, for the identification of Leishmania species from clinical samples. Primers were designed in the conserved region of the 18S ribosomal RNA (rRNA) gene; amplification was visualized by the pre-amplification addition of fluorescent detection reagent (FDR) and a simple UV lamp. By using a reverse-transcriptase step, the system detected infections between 10 and 100 parasites per ml. The assay was tested on a range of nucleic acid extracts from Leishmania species, visceral

leishmaniasis (VL) patients from Sudan, and cutaneous leishmaniasis (CL) patients from Suriname. The sensitivity of RT-LAMP from the blood of VL patients was 83% (N = 30) compared with microscopy of bone-marrow and lymph-node aspirates; for CL patients the observed sensitivity was 98% (N = 43). The potential to use LAMP as a diagnostic tool for leishmaniasis is significant.

3.11 Sensitivity, Specificity and Predictive Values of Diagnostic Tests

When assessing the accuracy of a diagnostic test, one must consider these components of screening test. They are expressed as percentages. Sensitivity and specificity are usually determined by applying the test to one group of persons having the disease, and to a reference group not having the disease. Sensitivity and specificity, together with 'predictive accuracy' are inherent properties of a screening test (Park, 2002).

Screening test results	Diagnosis		Total
	Diseased	Not diseased	
Positive	a (true positive)	b (false positive)	a+b
Negative	c (false negative)	d (true negative)	c+d
Total	a+c	b+d	a+b+c+d

The letter ‘a” denotes those individuals found positive on the test who have the condition or disorder being studied (i.e., true positives). The group labeled “b” includes those who have a positive test result but who don’t have the disease (i.e., false positives). Group “c” includes those with negative test results but who have the disease (i.e., false negatives). Finally, those with negative results who don’t have the disease are included in group “d” (i.e., true negatives).

Evaluation of a screening test

The following measures are used to evaluate a screening test:

a) Sensitivity = a/ (a+c) x 100

b) Specificity = d / (b+d) x100

c) Predictive value of positive test = a / (a+b) x100

d) Predictive value of negative test = d/ (c+d) x100

e) Percentage of false negative = c/ (a+c) x100

f) Percentage of false positive = b/ (b+d) x100

Source: (Park, 2002).

1. Sensitivity: This term sensitivity was introduced by Yerushalmy in 1940s as a statistical index of diagnostic accuracy. It is the capacity to correctly identify diseased individuals in a population that is "true positives". The greater the sensitivity, the smaller the number of unidentified cases (false negative)

2. Specificity: It is the capacity of a test to identify correctly those individuals who are free of the disease that is "true negative". The greater the specificity, the fewer "false positive" will be included.

3. Predictive accuracy: In addition to sensitivity and specificity, the performance of screening test is measured by its "predictive values" which reflects the diagnostic power of the test. The predictive accuracy depends upon sensitivity, specificity and disease prevalence. The "predictive values of a positive test" indicates the probability that a patient with a positive test result has, in fact, the disease in the question. The more prevalent a disease is in a given population, the more accurate will be the predictive value of a positive screening test. The predictive values of a positive result falls as disease prevalence declines (Park, 2002).

4. False Negative and Positive: Whereas the epidemiologist thinks in terms of sensitivity and specificity, the clinician thinks in terms of false negatives and false positives. The term "false-negative" means that patients who actually have the disease are told that they do not have disease. It amounts to giving them a "false reassurance". A screening test, which is very sensitive, has few "false negatives". The lower the sensitivity, the larger will be the number of false negatives (Park, 2002).

CHAPTER IV

4. MATERIALS AND METHODS

4.1 MATERIALS

A complete list of bacteriological media, reagents, chemicals, equipments, glass wares and miscellaneous materials used in the study are given in appendix I

4.2 Methods

4.2.1 Study Site

This study was carried out from February 2009 to March 2010 at the National TB Reference Lab of German-Nepal Tuberculosis Project (GENETUP), Kalimati, Central Department of Microbiology, Tribhuvan University (CDMTU), Everest International Clinic and Research Center (EICRC), Kathmandu in collaboration with Osaka Perfectural Institute of Public Health, Japan.

4.2.2 Study population

Study population covers all the patients visiting GENETUP of age variation 0 to above70 from February 2009 to March 2010. It includes both male and female gender for observing statistical significances. The following two groups of specimens were included in this study.

1) Study group A

These were the **suspected pulmonary TB patients** visiting German-Nepal Tuberculosis Project (GENETUP) hospital. In this group, a total of 103 sputum specimens from patients were collected.

2) Study group B

These were the **Follow up patients (Confirmed TB patients)** visiting GENETUP hospital for the drug efficacy. In this group, a total of 32 specimens from patients were collected.

4.2.3 Data collection

Data collection was done from each patient by interview through questionnaire given in the appendix VI. Clinical history (name, age, sex, signs and symptoms, present and past history of treatment of tuberculosis) of patients were collected. During sample and data collection, all research objectives had been briefed and then verbal consent had been taken from each study participants.

4.2.4 Sample collection

Sputum was the sample of choice in this study. For the new patients suspected of pulmonary tuberculosis, three sputum samples were taken, one "on the spot" during the time when patients visited to the hospital, followed by a two sputum sample on the next days, first at the early morning and third on the spot. All the samples were collected in leak proof, wide mouth, and transparent, sterile and stopper plastic container. Among triplicate sputum samples collected at GENETUP, only third sample per patient was included in this study. 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.

4.2.5 Sample evaluation

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

4.2.6 Sample processing

Before sample processing, the entire sample was examined for the visual appearance to have a desired sample and then further processed for microscopy and culture on the same day of collection.

4.2.6.1 Sputum smear microscopy

An appropriate amount of the sample was taken from the container with the sterile cotton swab and transferred to the clean, grease free slide. The sample was spread on the slide over an area of approximately 2.0 by 1.0 cm and made it thin enough to be

able to read through it. Then smear was allowed to air dry for 15 minutes without heating. Thereafter, the smear was heat fixed passing the slide over the Bunsen burner three to four times with the smear uppermost and allowed to cool before staining. These overall processes were performed inside a safety cabinet II.

Among the several methods of determining acid-fast nature of mycobacteria, fluorochrome method was included 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 (Appendix III). After washing with distilled water, the smear was allowed to air-dry and examined directly under the UV microscope. The organisms appeared as yellowish rods against a dark background. The microscopy result was interpreted on the basis of WHO.

Table 1: Reporting Criteria of Sputum Smear Microscopy

No. of Acid Fast Bacilli (AFB) observed per field	**Report**
No AFB / 300F	Negative
1-9 AFB / 100F	Positive (Record exact no. of AFB per 100 Visual Field (VF)
10-99 AFB / 100VF	Positive (1+)
1-10 AFB / VF in at least 50 VF	Positive (2+)
>10 AFB / VF in at least 20 VF	Positive (3+)

Source: WHO, 1998

4.2.6.2 Sputum decontamination and concentration

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

The sediment was harvested after discarding the supernatant and was used for culture and LAMP.

4.2.6.3 Sputum Culture

For culture, 0.1 ml of concentrated sputum was inoculated into each of two LJ slants. The slants were laid with medium face up for 30 minutes to allow the bacteria to adhere to the surface of the medium. The tubes were incubated at 37^{o}C for up to 8 weeks. The tubes were examined on 7th 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. Contamination was recorded when it was apparent. Negative report was given when no colonies appeared after observing weekly for 8 weeks. Grading of culture is given as

Table 2: Grading of culture result

Reading	**Report**
No growth	Negative
1-19 colonies	Positive (Number of colonies)
20-100 colonies	Positive (1+)
100-200 colonies	Positive (2+)
200-500 colonies (almost confluent growth)	Positive (3+)
> 500 colonies (confluent growth)	Positive (4+)
Contamination	Contaminated

Source: WHO, 1998

4.2.6.4. Sample treatment for LAMP

About 200 µl of the concentrated and decontaminated material was transferred in the effendorf tube and equal volume of 50 mM Tris HCl of pH 8.3 was added .Then entire specimen was centrifuged at 12,000 rpm for 15 minutes and treated at 80^{o}C for 10 minutes in a heat block to inactivate and cooled to room temperature. Then 400 µl of chloroform:isoamyl alcohol (1:3) was added and mixed by vortex and stored in refrigerator. Then supernatant was discarded and the pellet was resuspended in 200 µl of 50 mM Tris HCl of pH 8.3.

DNA Extraction

Freezing and thawing method was used in this study for DNA extraction, modified form of freeze and boil method standardized by Wood and Cole, 1989.

The treated sample in the eppendorf tube was treated at 95^{o}C for 10 minutes in hot water bath.Then,3 times freezing and thawing (freezing at -20^{o}C for 20 minutes and thawing at room temperature for 20 minutes) was repeated. Thus prepared DNA was used for LAMP.

4.2.6.5 Loop-Mediated Isothermal Amplification (LAMP)

Loop-Mediated Isothermal Amplification (LAMP) was performed with mycobacterium DNA extracted from the concentrated sputum sample was used for LAMP with six primers specially designed targeting *gyrB* gene, following the protocol previously standardized by Iwamoto in Japan (Iwamoto et al., 2003).

a. LAMP reaction

Number of cycle: LAMP was performed in a total 25 « l reaction mixture and resulting mixture was then incubated for one hour in thermocycler. Denaturation, Primer annealing and Extension steps were not required for this technique.

Temperature: The whole reaction was performed at isothermal temperature i.e. 64^{o}C for one hour.

Primers and Enzymes: All together six primers were used during the study. These were: outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and loop primers (loop F and loop B). They recognize eight distinct regions on the target DNA.LAMP was performed in a total 25 « l reaction mixture containing 10 x LAMP buffer, 14 mM dNTPs, 5 M betaine, 100 mM $MgSO_4$, primer mix (F3, B3, BIP, FIP, loop F, loop B), *Bst* DNA polymerase, distilled water, DNA samples, and fluorescent detection reagent for the direct detection of LAMP amplicons in the reaction tube with naked eye. One negative control and one positive control (buffer spiked with DNA) were included in each run.

For 1 sample/tube	
Mixture Components	Volume (25 « l)
10 x LAMP buffer	2.5 « l
14 mM dNTPs	2.5 « l
5 M Betaine	4.0 « l
100 mM $MgSO_4$	1.5 « l
Primer Mix (85-2L)	2.0 « l
Bst DNA polymerase (8,000 U/ml)	1.0 « l
D/W	7.5 « l
Fluorescent Dye	1.0 « l
DNA sample	3.0 « l

↓

Thermal cycle at 64^0 C for 60 min.

↓

Visual inspection of LAMP amplified products against controls.

Figure2: Standard Operating Procedure for LAMP

b. Observation of Results

LAMP amplicons in the reaction tube were directly detected with the naked eye by observing the change in color of the solution containing reaction mixture with a fluorescent dye that give distinguished color. Calcein in fluorescent detection reagent was initially combined with manganese ions to achieve the quenching effect. The amplification generates the by-product, pyrophosphate ions, which will bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. From this feature, the presence of fluorescence can indicate the presence of target gene and visual detection can be achieved it fluoresces green indicating target gene amplification.

c. Interpretation of the Results

The solution turned green in the presence of amplification product indicating positive result, while it remained orange with no amplification indicating negative result.

4.2.6.6 Statistical Analysis

For the comparison of LAMP with standard culture as well as microscopy, the statistical analysis of tests were carried out by calculating sensitivity, specificity, positive and negative predictive values, percentage of false negative and percentage of false positive. Data analysis was done by using SPSS software.

4.2.6.7 Safe disposal

Used eppendrof tubes and pipette tips were first treated with sodium hypochlorite solution and then discarded in the plastic containers in sealed condition. Culture test tubes and glass slides were sterilized by autoclaving and then discarded in plastic containers in sealed condition.

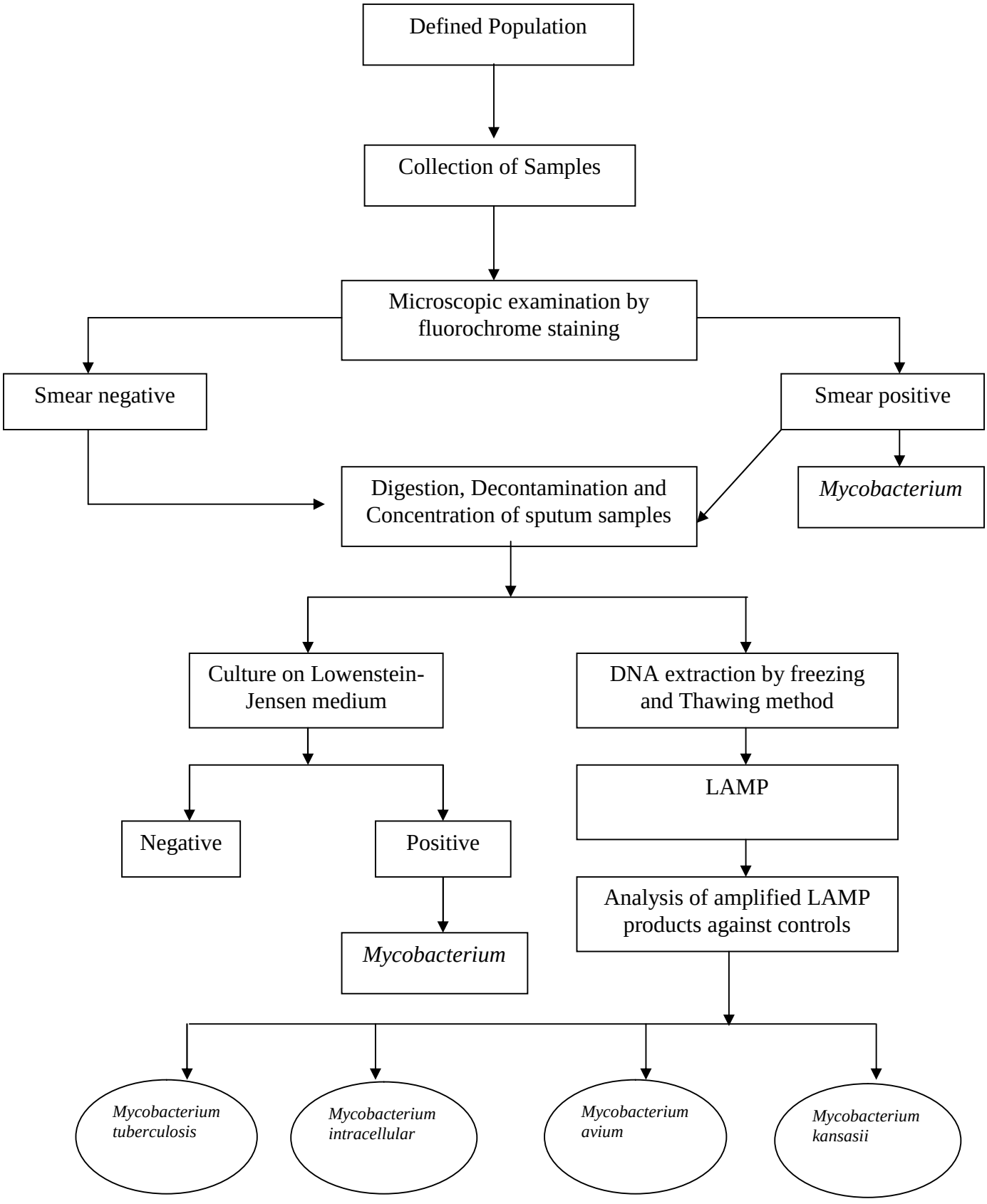


Figure 3: Flow Chart of Methodology

CHAPTER V

5. RESULTS

A total of 135 sputum samples from two different study groups were collected in this study. Out of 135 samples, 103 were from new suspected tuberculosis patients and 32 from follow up patients and Multidrug Resistant TB patients. All the collected samples were examined by microscopy, culture and LAMP. The results obtained by culture and microscopy were used for evaluating the specificity and sensitivity of the LAMP over the sputum smear microscopy and culture.

5.1 Laboratory Results

In this study, a total of 135 sputum samples were collected from two different study groups.

5.1.1 Study group A

This group includes 103 sputum samples from suspected tuberculosis patients visiting National TB Reference Lab of GENETUP, Kalimati.

5.1.1.1 Microscopy

From this group, all sputum specimens were examined by fluorochrome staining. Out of 103 (100%) sputum specimens, 35 (33.9%) were positive and remaining 68 (66.1%) were negative by fluorochrome staining.

5.1.1.2 Culture

Out of 103 (100%) sputum specimens examined by culture according to culture facility at the laboratory. 42 (40.7%) were positive by culture while remaining 61 (59.2%) sputum specimens were negative.

5.1.1.3 LAMP

Out of 103 (100%) sputum samples examined by LAMP for *M. tuberculosis*, *M. intracellular*, *M. avium* and *M. kansasii*, 44 (42.7%) were positive with LAMP and remaining 59 (57.2%) were negative.

Out of 44 LAMP positive cases 44 were positive with *M. tuberculosis* primer and *M. intracellular primer*. *M. avium* and *M. kansasii* cases were not found in this study

Among 103 sputum specimens examined by Microscopy, culture and LAMP, 33.9% specimens were positive by fluorochrome staining. Similarly, 40.7% sputum specimens were positive by culture on L-J media and 42.7% specimens were positive by LAMP.

Table 4: Results of 103 sputum specimens with microcopy, culture and LAMP

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
35 (33.9%)	68 (66.1%)	42 (40.7%)	61 (59.2%)	44 (42.7%)	-	-	-	44 (42.7%)	59 (57.2%)

5.1 .2 Study group B

This group includes 32 sputum specimens from follow up and Multi Drug Resistant TB patients visiting National TB Reference Lab of GENETUP, Kalimati. From this group all sputum specimens were examined by Microscopy, culture and LAMP.

5.1.2.1 Microscopy

All the sputum specimens from study group B were examined by fluorochrome staining. Out of 32 (100%) specimens, 25 (78.1%) were positive and 7 (21.8%) were negative by fluorochrome staining.

5.1.2.2 Culture

Out of 32 (100%) sputum specimens examined by culture on L-J medium, 26 (81.2%) were positive by culture while remaining 6 (18.7%) were negative.

5.1.2.3 LAMP

Out of 32 (100%) sputum specimens examined by LAMP for *M. tuberculosis, M. intracellular, M. avium* and *M. kansasii*, 27 (84.3%) were positive by LAMP and

remaining 5 (15.6%) were negative by LAMP. Out of 27 LAMP positive cases 26 were positive with *M. tuberculosis* primer while 1 was positive with *M. intracellular* primer.

Among 32 sputum specimens examined by microscopy, culture and LAMP, 78.1% specimens were positive by microscopy, 81.2% specimens were positive by culture on L-J medium and 84.3% specimens were positive by LAMP.

Table 5: Results of 32 sputum specimens with microcopy, culture and LAMP

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
25 (78.1%)	7 (21.8%)	26 (81.2%)	5 (15.6%)	26 (81.2%)	1 (3.1%)	-	-	27 (84.3%)	5 (15.6%)

5.1.3 Results of total sputum specimens

A total of 135 (100%) sputum specimens (103 from study group A and 32 from study group B) were included in this study for comparative study of Microscopy, culture and LAMP. Among them, 60 (44.4%) were microscopy positive. Similarly 68 (50.3%) sputum specimens were positive by culture and 71 (52.5%) sputum specimens were positive by LAMP. Among the total 71 (100%) LAMP positive cases, 70 (98.5%) cases were *M. tuberculosis* where as 1 (1.4%) cases were *M. intracellular*. *M. avium* and *M. kansasii* cases were not found from the samples included in this study

Table 6: Result of total 135 sputum samples with Microscopy, Culture and LAMP

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
60 44.4%)	75 (55.5%)	68 (50.3%)	67 (49.6%)	70 (51.8%)	1 (0.7%)	-	-	71 (52.5%)	64 (47.4%)

5.1.4 Distribution of Total Culture Positive sputum.

Out of 135 sputum samples examined by culture, 68 (n=100) samples were culture positive. Among them 55 (80.8%) were male and 13 (19.1%) cases were female. The highest numbers of culture positive cases were found in an age group 31-40 (26.4%), followed by 21-30 (19.1%), 41-50 (16.1%), 51-60 (16.1%), 10-20 (14.7%) and above 60 (7.3%).

Table 7: Age and Sex wise distribution of total culture positive cases

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	7	10.3	3	4.4	10	14.7
21-30	13	19.1	0	0	13	19.1
31-40	15	22.0	3	4.4	18	26.4
41-50	8	11.7	3	4.4	11	16.1
51-60	10	14.7	1	1.4	11	16.1
Above 60	2	2.9	3	4.4	5	7.3
Total	55	80.8	13	19.1	68	100.00

5.1.5 Distribution of Total LAMP Positive Sputum with *M. tuberculosis* Primer.

Out of total 71 LAMP positive samples, 70 (n=100%) were *M. tuberculosis.* Among them, 80 % (n=56) were male and 20 % (n=14) were female. Among them the highest number of cases was found in an age group 31-40 (25.7%), 21-30 (20%), 41-50 (18.5%), 51-60 (15.7%) and 10-20 (14.2%) and above 60 (5.7%). Lowest numbers of cases were found in the age group 60 (5.7%).

Table 8: Age and Sex wise distribution of total *M. tuberculosis* cases

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	7	10	3	4.2	10	14.2
21-30	14	20	0	0	14	20
31-40	15	21.4	3	4.2	18	25.7
41-50	10	14.2	3	4.2	13	18.5
51-60	8	11.4	3	4.2	11	15.7
Above 60	2	2.8	2	2.8	4	5.7
Total	56	80	14	20	70	100.00

5.1.6: Distribution of Total LAMP Positive Sputum with *M .intracellular* Primer

Out of total 71 LAMP positive samples, 1 (n=100) were found *M. intracellular* positive which was found in elderly female i.e. above 60 from study group B.

Table 9: Age and Sex wise distribution of total *M. intracellular* cases

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	0	0	0	0	0	0
21-30	0	0	0	0	0	0
31-40	0	0	0	0	0	0
41-50	0	0	0	0	0	0
51-60	0	0	0	0	0	50
Above 60	0	0	1	100	1	100
Total	0	0	0	100	1	100

5.1.7 Socio-Demographic and Disease Characteristic of culture positive patients

A total 135 patients were interviewed personally through structured questionnaire. Among them, 50.3% (68/135) were culture positive. Among the total culture positive (n=68), 66.1% (45/68) were male and 33.8% (23/68) were female.42.6% (29/68) were smoker, 57.3% (39/68) were non-smoker; 42.6% (29/68) had habit of taking alcohol and 57.3% (39/68) were non alcoholic; 57.3% (39/68) showed past history of TB treatment whereas 42.6% (29/68) showed absence of past history of TB treatment. 23.5 % (16/68) showed prior presence of TB in their family members where as 76.4% (52/68) showed absence of TB in their family members. Out of total culture positive patients, 61.7% (42/68) were immunized by BCG vaccination, whereas 38.2% (26/68) were non-vaccinated. While asking the patients about the symptoms of TB, 85.2% (58/68) had the symptoms of fever, 69.1% (47/68) had chest pain, 94.1% (64/68) had cough, and 75% (51/68) had haemoptysis respectively.

Table 10: Socio-demographic and disease characteristic of Culture positive patients (n=68)

Socio-demographic characteristics	No of patients	Percentage
Smoking		
Smoker	29	42.6
Non-Smoker	39	57.3
Alcohol		
Habit of drinking	29	42.6
Not	39	57.3
Family history of TB		
Present	16	23.5
Absent	52	76.4
BCG		
Vaccinated	42	61.7
Non-Vaccinated	26	38.2
Symptoms		
Fever	56	85.2
Chest pain	47	69.1
Cough	64	94.1
Haemoptysis	51	75
X-Ray		
Positive	65	95.5
Negative	3	4.4

5.2 Evaluation of tests:

Evaluation of clinical performance of LAMP for the rapid detection of *M. tuberculosis*, *M. intracellular*, *M. avium* and *M. kansasii* in sputum samples were determined by comparing LAMP with the gold standard culture and flurochrome staining.

Among 135 samples studied by microscopy and LAMP, 58 were positive by both tests, 2 were positive only in microscopy whereas 62 samples were negative in both tests and 13 were negative only in microscopy. With reference to microscopy, the sensitivity and specificity of LAMP was 96.6%and 82.6% respectively. Predictive value of positive test was 81.7%, predictive value of negative test was 96.8%, percentage of false negative was 3.3% and percentage of false positive was 17.3%.

Table 11: Comparison of LAMP with reference to microscopy

LAMP	Test and Results	Microscopy		Sensitivity	Specificity	PPV	NPV	False - ve	False +ve
		+ve	-ve						
	Positive	58	13	96.6%	82.6%	81.7%	96.8%	3.3%	17.3%
	Negative	2	62						

Where, PPV: positive predictive value, and NPV: negative predictive value.

Out of 135 sputum samples subjected to culture and LAMP for the diagnosis of tuberculosis and other atypical mycobacteriosis, 66 samples were positive by both tests and 2 were positive only in culture, among them 62 were negative by both tests,5 were negative in culture only. While comparing the LAMP result with culture as gold standard, the sensitivity of LAMP was 97.5%, specificity was 92.5%, predictive value of positive test was 92.9%, predictive value of negative test was 96.8%, percentage of false positive was 7.4% and percentage of false negative was 2.9%.

Table 12: Comparison of LAMP with reference to culture

LAMP	Test and Results	Culture		Sensitivity	Specificity	PPV	NPV	False - ve	False +ve
		+ve	-ve						
	Positive	66	5	97.5%	92.5%	92.9%	96.8%	2.9%	7.4%
	Negative	2	62						

Where, PPV: positive predictive value, and NPV: negative predictive value

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The current high level of interest in NTM diseases is the result of two major recent trends: the association of NTM infection with AIDS and the recognition that NTM lung disease is encountered with increasing frequency in the non-AIDS population. So major emphasis must be given in rapid and accurate identification of species of NTM for appropriate therapeutically options. For this purpose we used a novel nucleic acid amplification method, loop-mediated isothermal amplification method for detection of *M. tuberculosis* complex, *M. avium, M. intracellular* and *M. kansasii* in the sputum samples. The samples were subjected to fluorochrome staining for detection of the acid-fast bacilli followed by culture on Lowenstein- Jensen medium and LAMP for the detecting *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii*. Then the sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive of LAMP and microscopy were calculated in reference to culture methods of diagnosis as "Gold Standard".

Although acid-fast microscopy is simple, quick and inexpensive but it does not confirm TB diagnosis as well as mycobacteria other than M. tuberculosis is also AFB in the smear microscopic examination. In addition, a high bacterial load is needed in the specimen to render an AFB microscopy result positive. Though culture techniques have been estimated to detect as many as 10–1,000 viable mycobacteria per ml of specimen including species of bacilli, it is time consuming. Therefore, we need to have novel molecular techniques that combine the rapidity of microscopy ant the sensitivity of culture such as LAMP that can identify mycobacterium species. Even though this technique are not used routinely used in Nepal, some investigators reported its feasibility (Sohn et al., 2003; Poudel, 2005; Adhikari, 2007)

The Loop-Mediated Isothermal Amplification (LAMP) operation is quite simple. It starts with the mixing of the buffer primers, DNA lysates and DNA polymerase in

tube, and then the mixture is incubated at 64ºC for 1 hour. There is no necessity for heat denaturation of the template DNA. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of 64ºc. Visual judgment eliminates the need for any laboratories and time consuming post amplification operations such as hybridization and electrophoresis as well as the need for special equipment. LAMP assay is more advantageous than all of the currently available DNA probe methods in its simple operation and rapid reaction.

In this study, the LAMP was performed on sputum samples using species- specific primers for *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii* that were designed by targeting the *gyr* B gene sequences.

A total of 135 sputum samples belonging to two different categories were included in this study. Among the collected sputum samples, 103 were from new suspected pulmonary tuberculosis patients and 32 were from follow up MDR patients.

In this study, 68 (n=100) samples were culture positive. Among them, 55 (80.8%) were male and 13 (19.1%) cases were female and the result was found to be significant statistically. This result was in agreement with the report of Poudel (2005), Adhikari (2007) The highest numbers of culture positive cases were found in an age group 31-40 (26.4%), followed by 21-30 (19.1%), which is the economically most productive age group which is in agreement with Poudel (2005).

Among 60 (100%) PTB patients with a positive acid-fast stain on sputum smear, 57 were positive by chest X-ray while the remaining were negative. The X-ray positive results are due to cavities usually develops because of immune response to the tubercle bacilli leading to destruction of lung tissue. The X-ray negative result may be due to the people who don't have fully functioning immune system like in HIV cases, where there is less tissue destruction and hence lung cavitations. Another reason might be due to the patients who were suffered from bronchial or tracheal tuberculosis in whom infections might not spread to the lungs.

Out of 75 (100%) patients with negative acid–fast stain on sputum smear, 28 were positive in chest X-ray and the remaining were negative. Abnormalities on chest X-ray may be suggestive of, but are never diagnostic of PTB because a number of other

bacterial conditions (such as pneumonia or abscess) or non-bacterial processes (fungal diseases, carcinoma, sarcoidosis or pneumoconiosis) can produce similar images. A vast majority of patients (over 90%) with cavitary PTB are sputum smear positive (WHO, 2000).

While comparing the result of 135 sputum specimens with microscopy, culture and LAMP, 60 were microscopy positive. Similarly 68 sputum specimens were positive by culture and 71 sputum specimens were positive by LAMP. Among them 55 were found to be positive by flurochrome staining, culture and LAMP. Similarly 62 samples were negative by all diagnostic methods.

During the study period, a total of 135 sputum specimens were included to compare LAMP result with gold standard culture and Microscopy. Among the studied 13 sputum samples, 60 were fluorochrome staining positive. Out of 60 smear positive samples, 57 showed growths on Lowenstein-jensen medium but the remaining 3 showed no growth on this medium which were positive by LAMP. This may be due to the over decontamination of sputum sample which may kill the organism and thus these samples showed no growth on the culture medium. Similarly 75 sputum samples were fluorochrome staining negative. Out of them, 11 samples were positive while culture on LJ-medium and LAMP. This may be due to lower number of organism present in the sputum sample. Less than 5,000-10,000 organisms per ml are not detected through the microscopy but culture method can detect as approximately 10-100 bacilli per ml of sample.

By culture, 68 sputum samples showed growth on the Lowenstein-Jensen medium. Upon testing by LAMP, 66 culture positive samples were positive indicating high sensitivity of LAMP. Remaining 2 culture positive samples were negative by LAMP; this may be due to inefficient lysis of cells during freezing and thawing process for DNA extraction. The negative result may also be due to using primers targeting few species only. In this study we used primers targeting only four Mycobacterial species (*M. tuberculosis*. *M .avium*, *M. intracellular* and *M. kansasii)*. The mycobacteria grown on the culture media may be *M. xenopi*, *M. fortuitum* and other atypical mycobacteria which could not be detected by LAMP in this study.

Among 67 cultures negative sputum samples 5 were positive by LAMP, 3 culture negative samples, were positive by smear also. Most of these samples were from follow up patients who were under treatment. The possible cause of culture negative but LAMP positive result may be due to fast sputum conversion rate by culture than PCR among follow up patients due to effect of anti TB drug. Another possible cause for that type of result may be due to over decontamination of sputum specimens.

In this study, LAMP was performed by using primers targeting *M. tuberculosis*, *M. intracellular*, *M. avium* and *M. kansasii*. Among 71 LAMP positive samples, 70 were positive with *M. tuberculosis* primer and remaining 1 was positive with *M. intracellular* primer. None of the *M. avium* and *M. kansasii* cases were found during the overall study period. *M. intracellular* -pulmonary disease was found among elderly female (of age 65). The case was found in MDR follow up patient. This might be due to weak immunologic function of old age people and people with pre-existing lung disease.

While assessing the accuracy of the diagnostic test, sensitivity and specificity are very important components to be considered. In this study, LAMP has been compared with culture and microscopy taking them as reference methods. Further the accuracy of the test has also been evaluated by calculating predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive.

While comparing to microscopy, the sensitivity was 96.6% indicating high accuracy rate of LAMP and specificity of LAMP was 82.6%. The lower specificity of LAMP as compare with Microscopy may be due detection limit of organisms by Microscopy. Lower number of organisms (less than 5,000-10,000) in the sputum cannot be detected by smear microscopy which may be positive by LAMP. Predictive value of positive test was 81.7%, predictive value of negative test was 96.8%, percentage of false negative was 3.3% and percentage of false positive was 17.3%.

While comparing the LAMP result with culture as gold standard, the sensitivity of LAMP was 97.5%, specificity was 92.5%, predictive value of positive test was 92.9%, predictive value of negative test was 96.8%, percentage of false positive was 7.4% and percentage of false negative was 2.9%. This demonstrates the high sensitivity indicating high

accuracy rate of LAMP. In this study, LAMP showed lower specificity when compare with culture. The lower specificity of LAMP might be due to following reasons: The sputum samples which showed culture negative but LAMP positive results were mostly from follow up patients which were under anti TB drug therapy. Due to effect of this drug, sputum from these patients might be showed culture negative result faster than those from PCR indicating fast sputum conversion rate by culture than PCR. Hence from those patients culture negative sputum samples might be positive by LAMP. This may also be due to over decontamination of sputum samples which may kill the organism and ultimately gave negative result by culture but positive by LAMP. Percentage of false negative and percentage of false positive are also low indicating the higher accuracy of the test.

Paudel (2006), Adhikari (2008) evaluated the LAMP result with gold standard culture. LAMP demonstrates high sensitivity and specificity. With reference to microscopy, the sensitivity of LAMP was 98.1%, indicating high accuracy rate of LAMP and specificity was 67.1%. The lower specificity of LAMP as compare with microscopy may be due detection limit of organisms by microscopy. Lower number of organisms (less than 5,000-10,000) in the sputum cannot be detected by smear microscopy which may be positive by LAMP.

The finding of this study is in agreement by other literatures as given below. According to Iwamoto et al. (2003), the sensitivity study of LAMP on sputum sample using purified DNA indicated that the LAMP assay has a detection limit equivalent to that of Amplicor test. When they compared the results obtained from 66 sputum samples, 5 samples were positive by the Amplicor test but negative by LAMP. Four of the five samples were culture positive. None of the LAMP positive samples were negative by Amplicor. In this case, these results showed that the sensitivity of LAMP assay on sputum samples is slightly lower than that of Amplicor. They explained the discrepancy between the results obtained with the purified DNA and sputum might be due to the different sample sizes used in these two assays. For the Amplicor they used 50 µl of the DNA lysates, while for LAMP assay only 2.75 µl. On the final, they concluded that the rate of detection of mycobacterium in clinical samples can be increased when more compatible DNA extraction method for the LAMP assay is developed.

Boehme et al. (2007) reported that the sensitivity of LAMP for pulmonary TB in smear-positive, culture-positive specimens was 97.7% (173/177 specimens). In the small number of smear-negative, culture-positive specimens, the sensitivity of LAMP was 48.8% (21/43 specimens). The overall sensitivity of LAMP in the 220 culturepositive specimens was 88.2%. Of the culture positive specimens, 15 were reported to have visible heme, none of which were false negative by LAMP, suggesting that blood does not have an important inhibitory effect on the amplification or fluorescence detection. The specificity of LAMP in culture negative samples was 99.0% (500/505 specimens). Clinical follow-up of LAMP-positive, culture-negative patients with suspected TB was not performed, and no discrepant analysis was made. Amplification results were read at 30 min and at 40 min to determine whether the reaction time could be shortened.

Since LAMP is a novel nucleic acid amplification technique, there are very few publications reporting its use for direct detection of *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii in* sputum samples. So results of this study have been compared with investigators who applied LAMP for diagnosis of diseases other than tuberculosis

Hara-kudo et al. (2005) used LAMP assay to detect *salmonella* within 60 min. The 220 strains of 39 serotypes of *salmonella* subsp. *enterica* and 7 strains of *S. enterica* subsp. *arizonae* were amplified, but not 62 strains of 23 bacterial species other than *Salmonella.* The sensitivity of the LAMP assay was found to be 2.2 cfu / test tube using nine serotypes. The specificity was similar to that of a PCR assay, but the sensitivity of LAMP was greater.

Seto et al. (2008) used LAMP assay which provided markedly more sensitive and rapid detection of CT (Cholera toxin) -producing *V. cholerae* strains than conventional biochemical and PCR assays. The assay correctly identified 34 CT-producing *V. cholerae* strains, but did not detect 13 CT non-producing *V. cholerae* and 53 non-*V. cholerae* strains. Sensitivity of the LAMP assay for direct detection of CT-producing *V. cholerae* in spiked human feces was 7.8×10^2 CFU per g (1.4 CFU per reaction). The sensitivity of the LAMP assay was 10-fold more sensitive than that of the conventional PCR assay. The LAMP assay for detection of CT-producing *V. cholerae* required less than 35 min

with a single colony on thiosulfate citrate bile salt sucrose (TCBS) agar and 70 min with human feces from the beginning of DNA extraction to final determination.

6.2 Conclusion

In conclusion, the LAMP-based assay used in this study allows rapid and accurate identification of *M. tuberculosis complex, M. avium, and M. intracellulare* in sputum specimens as result were appeared within one hour. Among the different molecular diagnostic techniques, LAMP assay is more advantageous due to its easy operation without sophisticated equipment, it will be simple enough to use in small-scale hospitals, primary care facilities and clinical laboratories in developing countries if problems such as during sample preparation, nucleic acid extraction and cross contaminations are addressed.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

In this study, LAMP was performed with six species-specific primers designed by targeting gyrB gene for direct detection of *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii* in sputum samples. A total of 135 sputum samples belonging to two different categories were included in this study. Among the collected sputum samples, 103 were from new suspected pulmonary tuberculosis patients and 32 were from follow up MDR patients. These specimens were subjected to culture according to culture facility at GENETUP hospital.

While comparing the result of 135 sputum specimens with microscopy, culture and LAMP, A total of 135 (100%) sputum specimens (103 from study group A and 32 from study group B) were included in this study for comparative study of Microscopy, culture and LAMP. Among them, 60 (44.4%) were microscopy positive. Similarly 68 (50.3%) sputum specimens were positive by culture and 71 (52.5%) sputum specimens were positive by LAMP.

Among 103 sputum specimens examined by Microscopy, culture and LAMP, 35 (33.98%) specimens were positive and 68 (66.1%) negative by fluorochrome staining. Similarly, 42 (40.7%) sputum specimens were positive by culture and 61 (59.2%) negative on L-J media and 44 (42.7%) specimens were positive and 59 (57.2%) were negative by LAMP. On the other hand 7 (6.7%) microscopy negative samples were positive by culture and LAMP. Here, 2 (1.9%) samples were LAMP positive even though they were negative by culture and microscopy.

Among 32 (100%) sputum specimens examined by microscopy, culture and LAMP, 25 (78.1%) specimens were positive and 7 (21.8%) negative by microscopy, 26 (81.2%) specimens were positive and 6 (18.7%) negative by culture on L-J medium and 27 (84.3%) specimens were positive and 5 (15.6%) negative by LAMP. On the other hand 2 (6.2%) LAMP negative samples were positive by culture and microscopy, 4 (12.5%)

microscopy negative samples were positive by culture and LAMP, 3 (9.3%) culture negative samples were positive by LAMP and microscopy.

Among the total 71 (100%) LAMP positive cases, 70 (98.5%) cases were positive by *M. tuberculosis* primer where as 1 (1.4%) cases were *M. intracellular*. *M. avium* and *M. kansasii* cases were not found from the samples included in this study.

While comparing to microscopy, the sensitivity and specificity of LAMP was 96.6%and 82.6% respectively. Predictive value of positive test was 81.7%, predictive value of negative test was 96.8%, percentage of false negative was 3.3% and percentage of false positive was 17.3%.

While comparing the LAMP result with culture as gold standard, the sensitivity of LAMP was 97.5%, specificity was 92.5%, predictive value of positive test was 92.9%, predictive value of negative test was 96.8%, percentage of false positive was 7.4% and percentage of false negative was 2.9%.

7.2 Recommendations

Based on this study, the following recommendations have been made.

1) LAMP can be used for the diagnosis of M. tuberculosis in sputum samples because of rapidity, sensitivity and specificity even in clinical laboratories with no specific equipments.

2) LAMP assay can be used for identification of the species of mycobacteria as performed in this study for *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii*.

3) Few culture positive cases were negative by LAMP in this study therefore primers targeting other atypical mycobacteria such as *M. fortuitum, M. xenopi* are recommended for further study.

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APPENDIX-I

List of equipments and materials used during the study

A) Equipments

Autoclave	: German
Biological safety cabinet, class II	: German
Coagulator	: German
Centrifuge	: German
Dry heat block	: German
Distilling apparatus	: German
Incubator	: German
Microscope	: Olympus, Japan
Magnetic stirrer	: German
Microcentrifuge	: German
PH meter	: German
Refrigerator	: German
Sterilizer	: German
Thermocycler	: MJ Research, Inc. USA
Vortex shaker	: German

Pipettes and tubes

Eppendrof tubes
Micropipettes
Micropipette tips
PCR tubes

APPENDIX-II

Bacteriological Media

Lowenstein-jensen Medium (L-J Medium) (Carman and Christain, 1993)

This medium is used for the culture of mycobacterial species (except *M.leprae*)

Ingredients

i) Mineral salt solution	gm/ml
Potassium dihydrogen phosphate, anhydrous [KH_2PO_4]	2.40
Magnesium sulphate [$MgSO_4$]	0.24
Magnesium citrate, quadrihydrate [$Mg(C_6H_5O_7)_2.4H_2O$]	0.60
Asparagine	3.60
Glycerol	12
Distilled water	1000ml

These ingredients were dissolved in the water by heating and through mixing. The solution was boiled for 2 hours in a steamer and sterilized in an autoclave (German) at 121^{o}C and 15-lbs pressure for 20 minutes.

ii) Malachite green solution

Malachite green solution (2gm/dl) was prepared in sterile condition and the solution was placed in incubator at 37^{o}C for 2 hours that help to dissolve the dye.

iii) Egg solution

Egg solution was prepared by using fresh eggs (not more than 4 days old). Depending on the size of the eggs, 20-24 eggs are usually needed. The eggs were washed carefully with soap and warm water, using the brush. They were rinsed in running water for 30minutes and water was drained. Then eggs were placed in sterile tray and covered with sterile paper and left to dry for 2-3 hours. (This can be done by cleaning shells of eggs with methylated spirit and then burning off the excess spirit.)

Then thoroughly hands were washed with soap and warm water. Rinsed in running water, then with spirit and dried. The eggs were cracked with a sterile knife and emptied contents into a sterile beaker and beated with a sterile eggbeater.

These three solutions were combined as follows:

Mineral salt solution	600ml
Malachite green solution	20 ml
Beaten egg solution	1000ml

All were mixed and distributed in 5ml aliquots in sterile screw-capped tubes or bottles.

Tubes or bottles were lied on their sides and inspissated at 80-85^{o}C for 50 minutes. Medium can be left in the inspissator overnight and then inspissated again at 80-85^{o}C for 30 minutes in the next day.

APPENDIX III

Reagents / Chemicals

1. Reagents for fluorochrome staining

A.For Auramine solution

Auramine	1gm/litre
Phenol	30ml/litre
Distilled water	1000ml
Ethanol (95%)	100ml

B. 20% Sulphuric acid

H_2SO_4	200ml
Distilled water	800ml

C. 0.1% Methylene blue

Methylene blue powder	1 gm
Distilled water	1000ml

2. Solution for sample decontamination

A. NaOH solution

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

NaOH pellet 40 gm

D/W 100 ml

Working solution: 4%NaOH (500ml)

40%NaOH 50 ml

D/W 450ml

3. Solution for DNA extraction

B. Tris HCl

Stock solutioin: 1M Tris -HCl of pH 8.3 [$C_4H_{11}NO_3$, FW 121.1] (100 µl)

12.1 gm of Tris was taken and dissolved in 60 ml (pH as 10.7). The pH at 8.3 was maintained by adding HCl and final volume up to 100ml was made.

Working solution: 50 mM Tris-HCl of pH 8.3 (1000 µl)

1 M tris HCl of pH 8.3	50 µl
D/W	950 µl

4. Reagents and solution for LAMP

All the reagents, primers and solutions required for LAMP were provided by Osaka Perfectural Institute of Public Health, Osaka, Japan.

LAMP Buffer
$MgSO_4$
Betaine (N, N, N-trimethylglycine)
Deoxyribonucleoside triphosphate (dNTPs)
Bst DNA polymerase.
Primer
Template DNA
Fluorescence dye

4. Primers (Invitrogen T_m life technologies)

Primer: 1

Sequence (5' to 3': AGC ACG CTG TCA ATC ATG TA)

Molecular weight (µg/mol)	6102.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	222.5	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	27.4
T_m(1M Na^+)	66	n moles per OD	4.4
T_m(50mM Na^+)	45	OD's	11.50
%GC	45	µg's	315.38
Coupling Eff.	99%	n moles	50.6

Primer: 2

Sequence (5' to 3': GAA CAA TCC GGA GTT GAC AA)

Molecular weight (µg/mol)	6160.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	238.9	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	25.7

T_m(1M Na^+)	66	n moles per OD	4.1
T_m(50mM Na^+)	45	OD's	12.40
%GC	45	µg's	319.73
Coupling Eff.	99%	n moles	50.9

Primer: 3

Sequence (5' to 3': CCT TTA GGC CA TGT CTT TA)

Molecular weight (µg/mol)	6075.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	204.5	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	29.7
T_m(1M Na^+)	66	n moles per OD	4.8
T_m(50mM Na^+)	45	OD's	12.00
%GC	45	µg's	356.48
Coupling Eff.	99%	n moles	58.6

Primer: 4

Sequence (5' to 3': TGC ACA CAG GCC ACA AGG GA

Molecular weight (µg/mol)	6146.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	231.6	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	26.5
T_m(1M Na^+)	72	n moles per OD	4.3
T_m(50mM Na^+)	51	OD's	11.60
%GC	60	µg's	307.83
Coupling Eff.	99%	n moles	50.1

Primer: 5

Sequence (5' to 3': AGA GTT TGA TCC TGG CTC AG

Molecular weight (µg/mol)	6149.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	217.4	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	28.2
T_m(1M Na^+)	68	n moles per OD	4.6
T_m(50mM Na^+)	47	OD's	12.50

%GC	50	µg's	353.55
Coupling Eff.	99%	n moles	57.5

Primer: 6

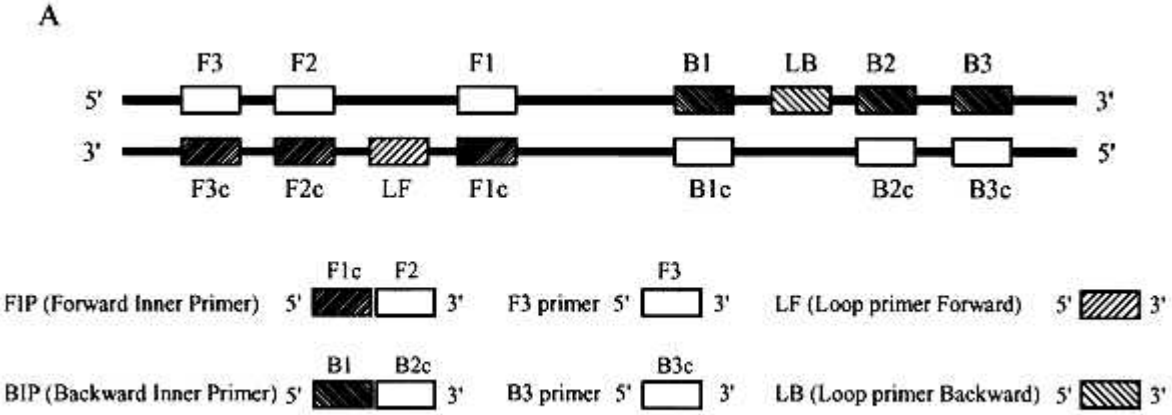
Sequence (5' to 3': ACC AGA AGA CAT GCG TCT TG

Molecular weight (µg/mol)	6127.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	225	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	27.2
T_m(1M Na^+)	68	n moles per OD	4.4
T_m(50mM Na^+)	47	OD's	10.80
%GC	50	µg's	294.10
Coupling Eff.	99%	n moles	47.9

APPENDIX IV

Primers used by Iwamoto et al., (2003)

A



B

MTB primers:

Gcgatatctggtggtctgca cggcgtcggc gtgtCggtgg ttaacgcgct aTccacccGg ctcgaagtcg
F3 F2 loop F
agatcaAgcG cgacgggtac gagtggtcTc aggtttAtga gaagtcggaa cccctggGCc tcaagcaagg
F1c B1 loop B
ggcGccgacc aaGaagacgg ggtcaacggT gcggttctgg gccgaccccG ctgttttcga aaccacgG
B2c B3c

MAV primers:

Gcctgaccat caacctcaCc gacgagCggg tgaccaacga agaGgtcgtc gaCgaggtgg tcagcgacAc
F3 F2 loop F
cgccgacgca cCcaagtcgg cgcaggagaa GgcggcggAa tcggctgcgc cgcataaGgT caagcaccgc
F1c B1 loop B
accttCcact accccggcgg Cctggtcgac ttcgtcaaAc acatcaatcg caCcaaaaac cccatccacc A
B2c B3c

MIN primers:

Acgaggtcgt cagcgataCc gccgacgcac cCaagtcggc ccaggaaaAg Gcggcggaat cgactGcGcc
F3 F2 loop F
acataaggtt aagcaccgCa cctccacta cccggcggt ctggtcgact Tcgtcaagca catcaaccgcA
F1c B1
ccaaGagcc cgatccagca gAgcatcatc Gacttcgacg gcaaaggtCc cggccacgag gtcgagatcg
loop B B2c
cgatgcagtg gaacggcgGc tactcggaat ccgtgC
B3c

Muniv primers:

Gcggtgtggg atggGcCcgc ggcctatcag Cttgttggtg gggtgacGgc ctaccaaggc gaCgaCgggt
F3 F2 loopF
agccggcctg agAgggtgtc cggccAcact gggactgaga tacggcCcAg actcctacgg gaggcaGcag
F1c B1 loop B
tggggaatat tgcacaatgg Gcgcaagcct gatgcAgcga cgccgcgtgg ggGatgacgg ccttcggG
B2c B3c

APPENDIX V

Statistical analysis of test

A) **Comparison of LAMP with reference to culture**

	Test and result	Culture		Total
		Positive	Negative	
LAMP	Positive	66 (a)	5 (b)	71 (a+b)
	Negative	2 (c)	62 (d)	64 (c+d)
	Total	68 (a+c)	67 (b+d)	135 (a+b+c+d)

Sensitivity = a/ (a+c) x 100 = 66/ (66+2) x100 = 97.5%
Specificity = d / (b+d) x100 = 62/ (5+62) x100 = 92.5%
Predictive value of positive test = a / (a+b) x100 = 66/ (66+5) x 100 = 92.9%
Predictive value of negative test = d/ (c+d) x100 = 62/ (2+62) x100 = 96.8%
Percentage of false negative = c/ (a+c) x100 = 2/ (66+2) x100 = 2.9%
Percentage of false positive = b/ (b+d) x100 = 5/ (5+62) x100 = 7.4%

B) Comparison of LAMP with reference to microscopy

	Test and result	Microscopy		Total
		Positive	Negative	
LAMP	Positive	58(a)	13 (b)	71 (a+b)
	Negative	2(c)	62 (d)	64 (c+d)
	Total	60 (a+c)	75 (b+d)	135 (a+b+c+d)

Sensitivity = a/ (a+c) x 100 = 58/ (58+2) x100 =96.6 %
Specificity = d/ (b+d) x100 = 62/ (13+62) x100 = 82.6%
Predictive value of positive test = a/ (a+b) x100 = 58/ (58+13) x100 = 81.7%
Predictive value of negative test = d/ (c+d) x100 = 62/ (2+62) x100 =96.8 %
Percentage of false negative = c/ (a+c) x100 = 2/ (58+2) x100=3.3%
Percentage of false positive = b/ (b+d) x100 = 13/ (13+62) x100 = 17.3%

APPENDIX VI

Questionnaires for data collection

Name:

Age: Sex: Weight:

Address:

1. Do you have habit of Smoking?

Yes No

2. Do you have habit of drinking alcohol? Yes No

3. Do you have family history of TB? Present Absent

4. Do you have past treatment history of TB? Yes No

5. Do you have scar of BCG vaccination? Yes No

6. Symptoms

i) Fever ii) Chest pain

iii) Cough iv) Haemoptysis

7. What is the report of x-ray? Positive Negative

APPENDIX VII

Results of total samples with microscopy,culture and LAMP

A) Laboratory tests for sputum samples of group A (n=103)

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
1	1b	52	M	Neg	Neg	Neg	Neg	Neg	Neg
2	2b	75	M	Pos	Pos	Pos	Neg	Neg	Neg
3	3c	27	M	Neg	Neg	Neg	Neg	Neg	Neg
4	4b	37	M	Neg	Neg	Neg	Neg	Neg	Neg
5	5c	60	M	Neg	Neg	Neg	Neg	Neg	Neg
6	6b	36	M	Pos	Pos	Pos	Neg	Neg	Neg
7	7c	41	F	Pos	Pos	Pos	Neg	Neg	Neg
8	8b	19	M	Neg	Neg	Neg	Neg	Neg	Neg
9	9b	57	M	Pos	Pos	Pos	Neg	Neg	Neg
10	10b	30	M	Neg	Neg	Neg	Neg	Neg	Neg
11	11c	55	M	Neg	Neg	Pos	Neg	Neg	Neg
12	12b	27	M	Pos	Pos	Pos	Neg	Neg	Neg
13	13c	45	M	Neg	Neg	Neg	Neg	Neg	Neg

14	14c	21	M	Neg	Neg	Neg	Neg	Neg	Neg
15	15b	37	M	Pos	Pos	Pos	Neg	Neg	Neg
16	16c	36	M	Pos	Pos	Pos	Neg	Neg	Neg
17	17b	74	M	Neg	Neg	Neg	Neg	Neg	Neg
18	18c	16	M	Neg	Neg	Neg	Neg	Neg	Neg
19	19b	35	F	Pos	Pos	Pos	Neg	Neg	Neg
20	20c	23	M	Neg	Pos	Pos	Neg	Neg	Neg
21	21b	28	M	Pos	Pos	Pos	Neg	Neg	Neg
22	22c	32	M	Pos	Pos	Neg	Neg	Neg	Neg
23	23b	24	M	Pos	Pos	Pos	Neg	Neg	Neg
24	24c	60	M	Neg	Neg	Neg	Neg	Neg	Neg
25	25b	23	M	Neg	Neg	Neg	Neg	Neg	Neg
26	26b	36	M	Pos	Pos	Pos	Neg	Neg	Neg
27	27c	41	M	Pos	Pos	Pos	Neg	Neg	Neg
28	28c	32	F	Neg	Neg	Pos	Neg	Neg	Neg
29	29b	33	F	Neg	Neg	Pos	Neg	Neg	Neg
30	30b	31	M	Neg	Neg	Neg	Neg	Neg	Neg

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
31	31b	29	M	Neg	Neg	Neg	Neg	Neg	Neg
32	32b	19	M	Neg	Neg	Neg	Neg	Neg	Neg
33	33c	17	F	Neg	Neg	Neg	Neg	Neg	Neg
34	34c	84	M	Neg	Neg	Neg	Neg	Neg	Neg
35	35b	24	M	Neg	Pos	Pos	Neg	Neg	Neg
36	36c	32	M	Neg	Neg	Neg	Neg	Neg	Neg
37	37b	45	M	Neg	Neg	Neg	Neg	Neg	Neg
38	38b	70	M	Neg	Neg	Neg	Neg	Neg	Neg
39	39c	45	F	Neg	Neg	Neg	Neg	Neg	Neg
40	40c	27	M	Neg	Neg	Neg	Neg	Neg	Neg
41	41c	56	M	Neg	Pos	Pos	Neg	Neg	Neg
42	42c	36	F	Neg	Neg	Neg	Neg	Neg	Neg
43	43c	19	M	Neg	Neg	Neg	Neg	Neg	Neg
44	44c	44	M	Neg	Neg	Neg	Neg	Neg	Neg
45	45b	73	M	Neg	Neg	Neg	Neg	Neg	Neg
46	46b	23	F	Neg	Neg	Neg	Neg	Neg	Neg
47	47b	40	M	Pos	Pos	Pos	Neg	Neg	Neg
48	48b	46	M	Pos	Pos	Pos	Neg	Neg	Neg
49	49c	40	M	Pos	Pos	Pos	Neg	Neg	Neg
50	50c	40	M	Neg	Neg	Neg	Neg	Neg	Neg
51	51c	31	M	Pos	Pos	Pos	Neg	Neg	Neg
52	52c	34	M	Neg	Neg	Neg	Neg	Neg	Neg
53	53c	67	M	Neg	Neg	Neg	Neg	Neg	Neg
54	54c	25	M	Pos	Pos	Pos	Neg	Neg	Neg
55	55c	49	M	Pos	Pos	Pos	Neg	Neg	Neg
56	56c	12	M	Neg	Neg	Neg	Neg	Neg	Neg
57	57c	30	F	Neg	Neg	Neg	Neg	Neg	Neg
58	58b	64	M	Pos	Pos	Pos	Neg	Neg	Neg
59	59b	27	M	Neg	Neg	Neg	Neg	Neg	Neg
60	60c	38	F	Neg	Neg	Neg	Neg	Neg	Neg

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
61	61b	41	F	Neg	Neg	Neg	Neg	Neg	Neg
62	62b	12	F	Neg	Neg	Neg	Neg	Neg	Neg
63	63c	42	M	Neg	Neg	Neg	Neg	Neg	Neg
64	64b	31	F	Pos	Pos	Pos	Neg	Neg	Neg
65	65c	42	M	Neg	Pos	Pos	Neg	Neg	Neg
66	66b	75	M	Pos	Pos	Pos	Neg	Neg	Neg
67	67b	53	M	Pos	Pos	Pos	Neg	Neg	Neg
68	68c	26	M	Neg	Neg	Neg	Neg	Neg	Neg
69	69c	21	M	Neg	Pos	Pos	Neg	Neg	Neg
70	70b	32	M	Pos	Pos	Pos	Neg	Neg	Neg
71	71c	31	M	Neg	Neg	Neg	Neg	Neg	Neg
72	72b	28	M	Pos	Pos	Pos	Neg	Neg	Neg
73	73b	60	M	Neg	Neg	Neg	Neg	Neg	Neg
74	74c	20	M	Pos	Pos	Pos	Neg	Neg	Neg
75	75c	57	M	Neg	Neg	Neg	Neg	Neg	Neg
76	76c	45	M	Neg	Neg	Neg	Neg	Neg	Neg
77	77b	60	M	Neg	Neg	Neg	Neg	Neg	Neg
78	78b	24	M	Neg	Neg	Neg	Neg	Neg	Neg
79	79b	6	M	Neg	Neg	Neg	Neg	Neg	Neg
80	80c	15	M	Neg	Pos	Pos	Neg	Neg	Neg
81	81c	16	M	Neg	Pos	Pos	Neg	Neg	Neg
82	82b	22	M	Neg	Neg	Neg	Neg	Neg	Neg
83	83c	29	M	Pos	Pos	Pos	Neg	Neg	Neg
84	84b	15	M	Pos	Pos	Pos	Neg	Neg	Neg
85	85c	35	M	Neg	Neg	Neg	Neg	Neg	Neg
86	86c	59	M	Neg	Neg	Neg	Neg	Neg	Neg
87	87b	27	M	Neg	Neg	Neg	Neg	Neg	Neg
88	88b	65	M	Neg	Neg	Neg	Neg	Neg	Neg
89	89b	63	M	Neg	Neg	Neg	Neg	Neg	Neg
90	90c	62	M	Neg	Neg	Neg	Neg	Neg	Neg

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result

						MTB	MIN	MAV	MK
91	91b	28	F	Pos	Pos	Pos	Neg	Neg	Neg
92	92b	40	M	Neg	Neg	Neg	Neg	Neg	Neg
93	93c	38	M	Neg	Neg	Neg	Neg	Neg	Neg
94	94c	35	M	Neg	Neg	Neg	Neg	Neg	Neg
95	95c	81	M	Neg	Neg	Neg	Neg	Neg	Neg
96	96c	17	M	Neg	Neg	Neg	Neg	Neg	Neg
97	97c	23	M	Neg	Neg	Neg	Neg	Neg	Neg
98	98b	38	F	Pos	Pos	Pos	Neg	Neg	Neg
99	99b	42	M	Pos	Pos	Pos	Neg	Neg	Neg
100	100b	41	M	Pos	Pos	Pos	Neg	Neg	Neg
101	101b	15	M	Pos	Pos	Pos	Neg	Neg	Neg
102	102c	34	M	Pos	Pos	Pos	Neg	Neg	Neg
103	103c	30	M	Pos	Pos	Pos	Neg	Neg	Neg

B)Laboratory tests of sputum samples for group B(n=32)

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
1	M05	22	M	Pos	Pos	Pos	Neg	Neg	Neg
2	M07	19	M	Neg	Neg	Neg	Neg	Neg	Neg
3	M06	34	F	Pos	Pos	Neg	**Pos**	Neg	Neg
4	M30	60	F	Pos	Pos	Pos	Neg	Neg	Neg
5	M31	65	F	Pos	Pos	Pos	Neg	Neg	Neg
6	M32	20	M	Pos	Pos	Pos	Neg	Neg	Neg
7	M25	24	M	Pos	Pos	Pos	Neg	Neg	Neg
8	M26	58	M	Pos	Pos	Pos	Neg	Neg	Neg
9	M27	44	M	Pos	Pos	Pos	Neg	Neg	Neg
10	M28	33	M	Pos	Pos	Pos	Neg	Neg	Neg
11	M29	40	M	Pos	Pos	Pos	Neg	Neg	Neg
12	M01	56	M	Neg	Pos	Pos	Neg	Neg	Neg
13	M02	54	M	Neg	Pos	Pos	Neg	Neg	Neg
14	M03	43	F	Neg	Pos	Pos	Neg	Neg	Neg
15	M22	55	M	Pos	Pos	Pos	Neg	Neg	Neg
16	M04	54	M	Pos	Pos	Pos	Neg	Neg	Neg
17	M10	55	F	Pos	Pos	Pos	Neg	Neg	Neg
18	M11	27	M	Neg	Pos	Pos	Neg	Neg	Neg
19	M12	22	F	Pos	Pos	Pos	Pos	Neg	Neg
20	M13	50	M	Pos	Neg	Pos	Neg	Neg	Neg
21	M14	49	M	Pos	Neg	Pos	Neg	Neg	Neg
22	M15	33	M	Pos	Neg	Pos	Neg	Neg	Neg
23	M08	49	M	Pos	Pos	Pos	Neg	Neg	Neg
24	M09	32	M	Pos	Pos	Pos	Neg	Neg	Neg
25	M20	54	M	Neg	Neg	Neg	Neg	Neg	Neg
26	M21	33	M	Pos	Pos	Pos	Neg	Neg	Neg
27	M22	19	M	Neg	Neg	Neg	Neg	Neg	Neg
28	M16	15	F	Pos	Pos	Pos	Neg	Neg	Neg
29	M17	17	F	Pos	Pos	Pos	Neg	Neg	Neg
30	M18	20	F	Pos	Pos	Pos	Neg	Neg	Neg
31	M20	58	M	Pos	Pos	Neg	Neg	Neg	Neg
32	M21	55	M	Pos	Pos	Neg	Neg	Neg	Neg

Photograph 1 : Culture of Mycobacteria on Lowestein-jensen medium

Photograph 2 : Visual judgement of LAMP
P: Positive control, 83: Sample (positive), 98: Sample (positive), 61: sample (negative), 73: Sample (negative), N: Negative control