CHAPTER I

1. Introduction

Tuberculosis, a well known disabling disease, remains a Global Peril and is still affecting millions of people in many parts of the world. TB is a worldwide pandemic, although the highest rate per capita are in Africa (28% of total TB cases), half of all new cases are in 6 Asian countries (Bangladesh, China, India, Indonesia, Pakistan and Philippines) (WHO, 2007).

Tuberculosis is an infectious disease commonly caused by bacterium *Mycobacterium tuberculosis*, which most commonly affects the lungs (Pulmonary TB) but can also affect the CNS (Meningitis), lymphatic system, circulatory system (Miliary TB), genitourinary system, bones and joints. (Tuberculosis, 1911).

1.6 million People died from TB in 2005, equal to an estimated 4400 deaths a day. TB is a disease of poverty; affecting mostly young adults in their most productive age. The vast majority of deaths are in the developing world, with more than half of all deaths occurring in Asia. TB disease if left untreated, each person with active TB infects on average 10-15 people every year (WHO, 2007). In Nepal, it is estimated that about 45% of total population is infected with TB, out of which 60% are in the productive age group. Each year, 44,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. Introduction of treatment by Directly Observed Treatment Short-course (DOTS) has already reduced the number of deaths; however 8,000-11,000 people continue to die every year from this disease (NTP, 2004).

Laboratory diagnosis of *Mycobacteria* currently depends on Acid-fast microscopy and culture of processed sputum samples (Kent and Kubica, 1985). Acid-fast microscopy is the fastest, easiest and least expensive tool for the rapid identification of patients with mycobacterial infection but the method is unable to distinguish within the mycobacterium genus (Salfinger and pfyffer, 1994). This method has low sensitivity and specificity as well as it requires organism load of 5,000-10,000 bacteria per ml (Woods and Washington, 1987). Presently, two types of acid-fast stains are used in

clinical mycobacteriology laboratories. One is carbol fuchsin (Ziehl-Neelsen (Zn) or Kinyoun staining methods) and the other is flurochrome (usually auramie or aurmine-rhodamine). The culture technique is the gold standard for the confirmatory diagnosis of presence of *Mycobacteria*, as it has high degree of sensitivity and specificity. 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) and liquid media (Middle brook 7H12 and other broths). A further advantage for culturing mycobacteria on solid media is that growth can be quantified, colony morphology and pigmentation can be examined and biochemical tests can be performed. Clinical laboratories hold cultures for 6 to 8 weeks to achieve the maximum sensitivity (kent and kubica, 1985). The radiographic appearance can also help in the detection of pulmonary mycobacteriosis but they do not allow etiological diagnosis. The diagnosis of pulmonary tuberculosis by X-ray is unreliable because other chest disease may resemble tuberculosis on X-ray (WHO, 2000).

Identification of Mycobacterial isolates in the species level is a trouble some job for clinical laboratories. The conventional biochemical tests for identification of Mycobacterial species are slow and time-consuming because of the slow growth of *Mycobacterium* on culture media. Similarly microscopy method is unable to distinguish within the mycobacterium genus. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method to overcome this problem in which reagents reacts under isothermal conditions with high specificity, efficiency, and rapidity.

Although first observed soon after Koch's discovery of the tubercle bacillus, Non Tuberculosis Mycobacterium (NTM) were not widely recognized as human pathogens until the 1950s, when several large series of patients with NTM lung disease were reported (Crow et al., 1957; Lewis et al., 1960 and Timpe et al., 1954). 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).

Loop-Mediated Isothermal Amplification (LAMP) is a method introduced by Notomi et al., 2000. 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). 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 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 has been designed to evaluate the use of LAMP in detection of Mycobacterial species including *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium 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 tubeculosis as well as other atypical Mycobacteria for effective treatment and case management.

CHAPTER II

2. Objectives

2.1. General objectives

Evaluation of Loop-Mediated Isothermal Amplification (LAMP) for diagnosis of pulmonary disease due to *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex and *Mycobacterium kansasii* in Nepal.

2.2. Specific objectives

- Evaluation of LAMP in diagnosis of *M. tuberculosis, M. avium, M. intracellular, M Kansasii*.
- To identify *M. tuberculosis, M. avium complex and M. kansasii* rapidly in the sputum samples using LAMP.
- Comparison of LAMP with culture and Sputum Smear Microscopy techniques for the diagnosis of tuberculosis in Nepal.

CHAPTER III

3. Literature Review

3.1 Diseases

Tuberculosis (abbreviated as **TB** for Tubercle Bacilli) is a common and deadly infectious disease caused by the *Mycobacterium tuberculosis.* Tuberculosis most commonly affects the lungs (as pulmonary TB) but can also affect the central nervous system the lymphatic system, the circulatory system, the genitourinary system, joints and even the skin (Tuberculosis, 1911).

Extra-pulmonary tuberculosis (EPTB) refers to disease outside the lungs. It is sometimes confused with non-respiratory disease. Disease of the larynx for example, which is part of the respiratory system, is respiratory but extra-pulmonary

At the time primary infection occurs blood or lymphatic spread of tubercle bacilli to parts of the body outside the lung may occur. In the fully immunocompetent host these bacteria are probably destroyed. If some immune deficit is present some may concentrate at a particular site where they may lie dormant for months or years before causing disease.

Bacteria may be coughed from the lungs and swallowed. By this route they may enter the lymph nodes of the neck or parts of the gastro-intestinal (GI) tract. Before milk was routinely pasteurised cattle infected with *M. bovis*, the bovine variant of tuberculosis could pass disease to humans who drank infected milk. Transmission by this route would also give rise to GI diseases. The commonest sites of infection are listed as follows Lymph glands and abscesses particularly around the neck. Orthopaedic sites such as bones and joints. The spine is affected in about half such cases. In women uterine disease is probably the most common while in men the epididymis is the site most frequently affected. Both sexes are affected by renal, ureteric or bladder disease equally. Abdomen. This may affect the bowel and or peritoneum. Meningitis, which may be rapidly fatal if not, treated in time Pericardium causing constriction to the heart Skin. Which can take a number of forms, most notably Lupus vulgaris where changes

of the facial skin was supposed to give patients a wolf-like appearance (http://www.priory.com/cmol/extratb.htm)

Two billion people, equal to one third of the world's population has the TB bacterium in their bodies and new infections are occurring at a rate of one per second .One in ten people infected with TB become sick with active TB in their life time (WHO, 2007) .TB is a leading killer among HIV-infected people with weakened immune system, about 200 000 people living with HIV/AIDS die from TB every year, most of them being in Africa (WHO, 2007).

Worryingly, HIV/AIDS and the neglect of TB control programme, drug-resistant strains of TB are also emerging (American thoracic society, 1990). 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).

Chronic pulmonary disease is the most common localized clinical manifestation of NTM (Falkinham, 1996 and wolinsky, 1979). *Mycobacterium avium* complex (MAC), followed by *M. kansasii*, are the most frequent pathogens causing lung disease. Other atypical pathogens occasionally causing pulmonary disease include *M. abscessus, M. fortuitum, M.szulgai, M.simiae, .M. xenopi, M.malmoense, M. celatum, .M. asiatium and M. shimodii*. The patients with chronic lung disease due to NTM are generally older adults. Except for patients with cystic fibrosis, children develop this form of NTM disease. Some NTM patients have a history of underlying chromic lung disease. The interpretation of NTM in the sputum of HIV-positive patients presents a particular problem, as these patients are frequently infected with NTM without evidence of pulmonary disease. Such infection may be transient, but it may also reflect disseminated NTM disease or sub clinical NTM pulmonary disease. (Wolinsky, 1979).

3.2 Mycobacteria

Organisms belonging to the genus mycobacterium are very thin, rod-shaped (0.2-0.4×2 to 10µm) and non motile. They are aerobic, non capsulated and resist decolorization by acidified alcohol (Forbes et al., 2000). 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%)[Good and Shinnick,1998] 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 an unusual cell wall structure that contains N-glycolylmuramic acid instead of N-acetylmuramic acid and has very high lipid content. Because of this cell wall structure, Mycoacteria are difficult to stain with commonly used basic aniline dyes, such as those used in the Gram stain (Forbes et al., 2002). They grow more slowly than most other human pathogenic bacteria because of their hydrophobic cell surface. Because of this hydrophobicity, organisms tend to clump, so that nutrients are not easily allowed into the cell. Growth is slow with colonies become visible in 2 to 60 days at optimum temperature (Forbes et al.,2000)

There are 71 recognized species in the genus Mycoacteria, which produce spectrum of infection in human and animals (Shinnick and Good. 1994). Clinically Mycobacteria are divided into two major groups based on the fundamental differences in epidemiology and association with disease: those associated with *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum)* and others *non-tuberculous mycobacteria* (NTM). The latter group has been several collective names-atypical, anonymos, tuberculoid and Mycoacteria 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- 0.5 µm (cheesbrough, 1989). The organism divides every 16 to 20 hours, this is extremely slow compared to other bacteria. In contrast, one of the fastest growing bacteria is a strain of *E. coli* that can divide roughly every 2 minutes. *M. tuberculosis* complex (MTB) is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either. If a Gram-stain is

performed, MTB either stains very weakly Gram-positive, or not at all (Forbes et al., 2000).

3.2.2 *Mycobacterium avium* complex and *Mycobacterium kansasii*

Mycobacterium 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 *Mycoacterium 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 also a non-sporing, non-capsulate, straight rod. Morphologically, it is similar to that of *M. tuberculosis*. It belongs to a group of Mycobacteria referred to as photochromogenic because they produce pigmented colonies when grown in presence of white light but not when incubated in the dark. 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

Tuberculosis is transmitted from person to person via the respiratory route. It is transmitted mainly by droplet infection and droplet nuclei containing viable virulent organism generated by sputum positive patients with pulmonary tuberculosis during coughing, sneezing and vocalizing. Coughing generates the large number of droplets of all size. One cough can produce 3,000-5,000 droplet nuclei (park, 2002). Tuberculosis is not transmitted by fomites such as dishes and other articles used by patients. The incubation period generally ranges form 3 to 6 weeks and some times up to months or years (Park, 2000).

Epidemiologic studies, skin test surveys and more recently DNA fingerprinting studies suggest that person-to-person transmission of pulmonary infection due to *M. avium, M. intracellular* and *M. kansassii* and other NTM are rare. Most persons are infected by

environmental NTM. Airborne NTM may play an important role for the source of infection in respiratory disease (Portaels,1995).

3.4 Pathogenesis

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within alveolar macrophages (Houben et al., 2006). The primary site of infection in the lungs is called the Ghon focus. Dendritic cells, which do not allow replication, although these cells can transport the bacilli to local lymph nodes, pick up bacteria. Further spread is through the bloodstream to the more distant tissues and organs where secondary TB lesions can develop in lung apexes, peripheral lymph nodes, kidneys, brain and bone (Herrmann et al., 2005)

Tuberculosis is classified as an infection of the granulomatous inflammatory conditions. Macrophages, T-lymphocytes, B-lymphocytes and fibroblasts are among the cells that aggregate to form granuloma, with lymphocytes surrounding the infected macropohages. The granuloma functions not only to prevent disseminatation of the mycobacteria, but also provides a local environment for communication of cells of the immune system . Within the grauloma, T-lymphocytes (CD-4) secretes cytokines such as interferon gamma (IFN- γ), which activates macrophages to destroy the bacteria with which they are infected (Kaufmann, 2002). T-lymphocytes (CD- 8) can also directly kill infected cells. If TB bacteria gain entry to the bloodstream from an area of tissue damage they spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues. The severe form of TB disease is most common in infants and the elderly which is called miliary tuberculosis. In many patients the infection waxes and wanes. Healing and fibrosis balance tissue destruction and necrosis. Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are in continuity with the air passages bronchi. This material may therefore be coughed up. It contains living bacteria and can pass on infection (Houben et al., 2006).

The mechanisms associated with the infection of the respiratory tract due to *M. avium* complex and *M. kansasii* are less known. *M. avium* complex infections of the lung

usually occur in individuals with chronic lung diseases (such as bronchictasis, emphysema etc.). One possibility for the establishment of chronic lung infection with colonization and infection of the bronchial mucosa is the development of biofilm. *M. avium* and *M. intracellular* are known to establish biofilms in municipal water system. (Falkinham et al., 2001).

Once *M. avium* complex reach the alveolar space, they come in contact with alveolar epithelial cells (alveolar macrophage). Recent studies have established that *M. avium* is capable of invading type II alveolar epithelial cells ad replicating within these cells (Bermudez and good man, 1996). Following phogocytosis, *M. avium* is observed within cytoplasmic vacuoles that do not acidify. *M. avium* phagosome does not mature and does not fuse with lysosomes. Other mechanisms that allow *M. avium* survival in marophages is that it shifts its metabolism with up-regulation of the enzyme Isocitrate lyase (zu et al., 1999), which is part of glycosylate bypass. Macrophages infected with *M. avium* undergo apoptosis and necrosis (Fratazzi et al., 1997, and Bermudez et. al., 1999). The bacteria can exit the apoptotic moraphage and infect another macrophage (Bermudez et al., 1997).

M. kansasii infections of the lung are likely acquired by inhalation of the organism. Once inside the lung, the bacteria attach to CR_3 (CD 11b/ CD18) on resident alveolar macrophages and are internalized into phagosomal compartments that do not fuse with lysosomes on produce super oxide ($O2^-$). 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 of Infection

Transmission of tuberculosis occurs primarily within household and other group of people living in close proximity. Such as prisoners and residents of common lodging houses (Van Genus et al., 1975). Children under the age of 3 years are particularly susceptible to infection from household source cases (Grange, 1990). The risks of

transmission of infection from person with sputum smear negative PTB and with extra pulmonary TB is low (WHO, 2004).

Tubercle bacilli are necessary, but not a sufficient cause of tuberculosis. The risk of developing tuberculosis largely depends upon the integrity of the cellular immune system (Rieder, 1995). There are multitude risk factors, which increase the risk of progression from sub-clinical infection to overt disease. These factors include HIV infection, diabetes mellitus, chronic lung disease, cancer, advanced kidney disease, malnutrition, alcoholism, heavy smoking, the elderly, poverty and use of intravenous drugs (Rieder et al., 1989).

The risk of developing *M. avium intracellular* pulmonary disease (MAC-PD) is largely on the immunocompromised people (i.e. individuals with HIV-AIDS) and individuals with pre-existing lung disease. Pre-existing lung diseases associated with MAC-PD are: smoking related chronic obstructive pulmonary disease, previous tuberculosis and other granulomatous lung disease, Radiation fibrosis, silicosis, cystic fibrosis, alcoholism, Bronchiectasis, Bronchogenic cancer, other causes (e.g., allergic broncho-pulmonary aspergillosis) (Rosenwieg et al., 1979 and Hornick et al., 1988)

Risk factors for *M. kansassi* infection include pneumoconiosis, chronic obstructive lung disease (COPD), previous mycobacterial disease, malignancy and alcoholism (Lillo et al., 1990; Bloch et al., 1998; Corbett et al., 1999 and Jocobson et al., 2000). The combination of HIV infection and silicosis is a potent factor for susceptibility to *M. kansassi* (Corbett et al., 1999).

3.6 Host Defense

M. tuberculosis pathogenicity is related to its ability to escape killing by marophage and induce delayed type hypersensitivity (Quinn, 1996). The pathogenicity depends upon the several components of cell wall. One of the main important component is the cord factor, a surface glycolipid, Lipoarabinomannan (LAM), a major heteropolysaccaride similar in structure to endotoxin of Gram negative bacteria, inhibits the macrophage activation by interferon-gamma (IFN-γ). LAM also induces macrophage to secrete TNF- α, which causes fever, weight loss and tissue damage and

IL-10, which inhibits IFN-γ production by T-cells (catran et al., 2000 and Fujimo et al.,).

Deficiencies in interferon (IFN-γ) production or the inability to express in IFN-γ or IL-12 receptor are associated with the development of infection by environmental mycobacteria mainly *M avium* (Altare et al., 1998 and Dorman et al., 1998). *M. avium* infection is counteracted in the host by non specific (innate) response as well as a specific immune response. Natural killer cells are the most relevant cells in the innate defense against *M. avium*. NK cells have been shown to secret TNF-α, INF-γ and GM-CSF in response to *M. avium* (Bermudez et al., 1991). NK cells released TNF-α can activate the antimycobacterial response in macrophage and the neutralization of NK cells TNF-α impairs the non-specific host response against the microorganism (Bermudez et al., 1991). IL-12 secreted by macrophages infected with *M. avium* stimulates NK cells to produce TNF- α (Bermudez et at., 1995).

Macrophages infected with *M avium* secrete IL-12 initially, but the production of IL-12 is blocked by the infection (Wagner et al., 2002). Inhibition of IL-12 production is not dependent on IL-10 (Wagner et al., 2002). It is possible that bacterial lipids released from infected cells may have a suppressor effect on uninfected cells (Atkinson et al., 2000). IL-12 production also disappears from the spleen later in the infection. IL-12 has been shown to be a key cytokine involved in host defense against *M. avium*, and its inactivation leads to significantly more *M. avium* disease (Castro et al., 1995 and Saunders et al., 1995).

3.7 Clinical Presentation

Clinical signs and symptoms develop in only a small proportion (5-10 percent) of infected healthy people (Mc Murray, 2001). Tuberculosis primarily affects the lower respiratory system and clinically these patients usually present with pulmonary disease and the prominent symptoms are chronic, productive cough, low-grade fever, night sweats, easy fatigability and weight loss.

Chronic pulmonary disease is the most common localized clinical manifestation of NTM (Falkinham, 1996 and wilinsky, 1979). Signs and symptoms MAC-PD and MK-

PD are variable and non-specific. They include chronic cough, sputum production and fatigue. Less commonly 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. (Jenkins et al., 1960)

3.8 Treatments and Control

Tuberculosis therapy generally consists of 6 to 9 month course of Isoniazid, rifampicin, streptomycin, thiocetazone, pyrazinamide and ethambutol (Apendix VIII). There are three main properties of anti-TB drugs: bactericidal activity, sterilizing activity and the ability to prevent resistance (Maher et al., 1997).

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. 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 mo, 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 of disseminated *M. avium* complex disease. Therapy in adults should include daily clarithromycin (500 mg twice a day) or azithromycin (250 to 500 mg), plus ethambutol 15 mg/kg per day (Appendix VIII). 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.,1996 and Dautzenberg et al.,1995)).

Treatment of *M. kansasii* pulmonary disease. A regimen of daily isoniazid (300 mg), rifampin (600 mg), and ethambutol (25 mg/kg for 2 mo, 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*(Appendix VIII). Clarithromycin or rifabutin will need to be substituted for rifampin in HIV-positive patients who take protease inhibitors (Wallace et al.,1997).

3.9 Diagnosis

Because of increasing frequency of NTM disease between both Immunocompromised and Immunocompetent, the rapid identification of tuberculosis and NTM diseases are key ingredients for effective treatment. 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).

3.9.1 Laboratory Diagnosis

3.9.1.1 Acid-fast Microcopy

Acid-fast microscopy is the fastest, easiest, and least expensive tool for the rapid identification of patients with mycobacterial infection (Salfinger et al, 1994 and Kent and kubica, 1985). Utilizing 'acid fast' property of mycobacteria carries out the microscopy. The acid-fast' staining procedure depends on the ability of mycobacteria to retain dye when treated with mineral acid or an acid-alcohol solution [Bloom, 1994]. Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One is carbol fuchsin (Ziehl-Neelsen (Zn) or Kinyoun staining methods) and the other is flurochrome (usually auramie or aurmine-rhodamine). In the carbol fuchsin (Ziehl-Neelsen) procedure, acid-fast organisms appear red against a blue background, while in the flurochrome procedures; the acid-fast organisms appear as fluorescent rods, yellow to oragne (the colour may vary with filter system used) against a pale yellow or orange background (WHO, 1998a). It is generally accepted that the fluorescent method should be given preference over the ZN and Kinyoun (Kent and Kubica, 1985 and Dunlap et al., 2000). Fluorochrome stains may stain other bacteria damaged by antituberculous drugs at a higher rate than carbol fuchsin and lead to a false positive result (Lipsky et al., 1984 and Gruft, 1999). This possibility should be considered when the specimen is from a patient on therapy. Because of a tendency

towards false-positivity with fluorochrome staining, good laboratory practice requires that any doubtful and smear-positive results on newly diagnosed patients should be confirmed by ZN staining (Metchock et al., 1999; salfinger and pfyffer, 1994; Kent and Kubica, 1985).

Whenever disease is suspected, three specimens must be collected for examination by microscopy. The examination of three specimens increases the predictive value of positivity of smear microscopy, reaching almost that of culture (chloride et al., 2000). Whenever possible, they should be obtained within twenty-four hours (WHOs 2000, Akhtar et al., 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; Peterson et al., 1999 and Wright et al., 1998). 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).

3.9.1.2 Culture:

A combination of different culture media is required to optimize the recovery of

mycobacteria from culture; at least one solid medium in addition to a liquid medium should be used (Forbes et al., 2002).

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 medias and into a liquid medium. At least three respiratory (sputum) cultures should be used for the initial evaluation. Lowenstein-Jensen is an excellent medium for recovery of *M. tuberculosis*, but is generally inferior to middlebrook agar as an all-purpose medium for both *M. tuberculosis* and *NTM*. (Heifets., 1994 and Wilson et. al, 1995). Cultures are examined weekly for growth. Contaminated cultures are discarded and reported as "contaminated, unable to detect presence of mycobacteria". Most isolates appear between 3 and 6 weeks; a few isolates appear after 7 or 8 weeks of incubation. After 8 weeks of incubation, negative cultures (those showing no growth) are reported, and the cultures are discarded. (Forbes et al., 2002).

Sputum culture to isolate mycobacteria is a highly sensitive diagnostic method that permits detection of a minimum of 10 to 100 viable bacilli per ml. of cultured material. The sensitivity of culture is excellent, ranging from 80% to 93%. (ATS-CDC, 2000; Dalovisio et al., 1996). Moreover, the specificity is quite high, at 98% (ATS-CDC, 2000). Thus, culture for the presence of mycobacteria is indispensable for the following reasons: culture is more sensitive for the detection of mycobacteria than acid-fast microscopy. It provides drug susceptibility tests and genotyping of particular cultured NTM can be used for epidemiologic purposes and to rule out cross contamination (Metchock et al., 1999, Von et al., 1994 and Mangione et al., 2001).

When growth appears, the rate of growth, pigmentation and colonial morphology are recorded. *M. tuberculosis* is an obligate aerobe, grows optimally at 37^0c (range: 25^0c to 40^0c) 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 (Forbes et al., 2000).

Mycobacterium avium complex (MAC) also are obligate aerobe, grows optimally at 37^0c and slow growing organism. On solid media (Ogwa and L-J). MAC produces smooth and non-adherent (they do not produce "cord factor") colonies. As they do not produce pigment, colonies are colorless. Wallenstein's medium, composed of egg yolk, 2.5% glycerin, malachite green and water, is an excellent medium for the recovery of NTM, particularly *M. avium* complex (MAC) (Forbes et al., 2000).

On solid media, *M. kansasii* produces same colonies as MAC but as a photochromogenic organism it produce pigment and the colonies are yellow in colored. Pigment is produce when grown in presence of white light but not when incubated in dark (Forbes et al., 2000).

The centers for disease control and prevention (CDC) recommend the use of both liquid and solid media in order to decrease the time to determine and to increase the yield of growth detection (Tenovar et al., 1993). For many years, the only culture system with the potential to decrease turnaround time was the BACTEC 460TB system. (Morgan et al., 1983). It has been shown, however, that the newly introduced Mycobacteria Growth Indicator Tube(MIGIT), BACTEC 9000 MB, MB redox , MB/Bac T and ESPII systems are suitable non-radiometric or fully automated alternatives to the radiometric BACTEC 460TB(Benjamin et al., 1998; Tortoli et al.,1998; and Pfyffer et al.,1997). It is noteworthy that , although all the alternative broth system showed a comparable sensitivity to the BACTEC 460TB, the MGIT system exhibited a significantly better recovery rate regarding the *M. avium* complex and other NTM (86% vs. 72% and 69% vs. 50% respectively) and the ESPII showed a higher recovery rate regarding the *M. avium* (94.6%vs 75.7%) (Tortoli et al., 1998 and Hanna et al., 1999)

3.9.2 Other Diagnostic Techniques for Pulmonary Mycobacteriosis

3.9.2.1 Radiographic Finding

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. In many patients with primary progressive tuberculosis and

those with HIV, radiographic findings are subtler and can include lower-lobe infiltrates or a miliary pattern. Miliary lesions, which are small gramulomatos, resemble millet seeds spread through the lungs (Bloom. 1994).

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). It is seen most commonly in white males and is often associated with other diseases, especially chronic pulmonary disease (Christensen et al., 1979 and Dutt et al., 1979). 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, 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)

3.9.2.2 Immunological Diagnostic Methods

a) Antigen- Protein Detection

The detection of microbial products or components has been used in recent years to diagnose infections caused by *M. tuberculosis*. For example, tuberculostearic acid is a fatty acid that can be extracted from the cell wall of mycobacteria and then detected by gas chromatography/mass spectrometry in clinical samples containing few mycobacteria. The presence of tuberculostearic acid in cerebrospinal fluid thought to be diagnostic for tuberculosis meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis (Savic et al., 1992).

(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. However, its performance in rapid diagnosis of active TB in disease-endemic areas is still unknown (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 o ELISA is the 38 kDs antigen from *M. tuberculosis* were also useful in ELISA (Kox,1996)

3.9.2.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) of mycolic acid of mycobacterium species has proved to be a rapid and reproducible tool to identify a wide range of known or unknown mycobacteria species either from culture or from sputum (Jost et al.,1995 and Butler et al., 1991). The fluorescent HPLC is more sensitive than the commonly used ultraviolet (UV) HPLC (Jost et al., 1995).

3.9.2.4 Molecular Techniques For The Diagnosis Of Pulmonary Mycobacteriosis

Diagnosis of infection due to nontuberculous mycobacterium is not easy; it must be distinguished from colonization or contamination by other nontuberculous mycobacteria. Molecular methods 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)

Nucleic acid amplification is one of the most valuable tools in virtually all life science fields, including application-oriented fields such as clinical medicine, in which diagnosis of infectious diseases, genetic disorders and genetic traits is particularity benefited by this new technique. In addition to the widely used PCR-based detection, several amplification methods have been invented. 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). In rare instances cross-reaction has been documented in the Acuprobe for the MTB complex with isolates of either *M. celatum* types 1 and 3 or *M. terrar* (Butler et al., 1994; Marytin et al., 1993 and somoskovi et al., 2000). Adherence to the proper hybridization temperature (60 and 61) was the most critical parameter (Somoskovi et al., 2000).

b) Polymerase Chain Reaction (PCR)

Kari Mullis polymerase chain reaction (PCR) was the first one and it is still the most popular amplification method. PCR 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. intraellular* 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). The specificity of the SDA reaction is based on the choice of primer to direct the DNA synthesis. When coupled with chemiluminescence-based hybridization detection system, the entire assay can be completed within 4th of obtaining processed specimen (Spargo et al., 1993). 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).

3.10 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 deoxyribo nucleoside 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 called SYBR Green I 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).

3.10.1 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).

3.10.2 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 (Fig.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

This 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.

3.10.3 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 and Chambelrlin 1996)

Deoxyribonucleoside triphosphate (dNTPs)

The dexyribonucleoside triphoshpates (dATP, dCTP, dTTP,dGTP) provide both the energy and nuelcoside 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 domain. 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.

SYBR Green I

LAMP amplicons in the reaction tube are directly detected with naked eye by adding SYBR Green I to the tube and observing the color of the solution. This provides great flexibility because no target-specific probes are required. The fluorescence intensity of SYBR Green is enhanced over 100-fold on binding to DNA which results in bright fluorescent DNA bands against a very dark background. Apart from its superior sensitivity, SYBR Green stain has other advantages over ethidium bromide because it is much less mutagenic, and it can be added directly to the DNA sample before electrophoresis.

3.10.4 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 by product of the LAMP reaction (Mori et. al., 2001). LAMP amplicons in the reaction tube are also directly detected with naked eye by adding SYBR Green I 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).

ds DNA binding by Syber Green I

The two positive charges in the core structure of Syber Green I are likely to contribute to the high binding affinity for dsDNA.. Intercalation is the main mechanism of SYBER Green I to bind double stranded DNA. The second binding mode of SYBER Green I is an interaction of SYBER Green I with the minor groove. Small molecules having aromatic rings connected by bonds with torsional freedom, and thus being able to fit into the helical curve of the minor groove, are reported to bind in the minor groove. The flexibility and torsional freedom of SYER Green I would allow for minor-groove binding. Also, the structure of SYER Green I should allow for electrostatic interaction of its cationic groups with the negative electrostatic potential in the minor groove as well as proximate van der Waals contacts within the boundaries of the minor groove. (Zipper et al., 2004). Large amount of amplicons when bind with SYBER Green I, it fluoresces green indicating target gene amplification.

3.10.5 Optimized Conditions for LAMP

Since hybridization of four primers to the target DNA in the initial step is critical for the efficiency of LAMP, Notomi et al. (2000) chose the sequences and size of the primers so that their melting temperature (T_m) fell in certain ranges. The F_2 and B2 sequences in FIP and BIP were chosen such that their T_m values fell between 60^0 C and 65^0C, the optimal temperature for *Bst* polymerase. The T_m values of Flc and Blc were set slightly higher than those of F2 and B2 in order that a looped out structure formed immediately after release of the single-stranded DNA from the template. Furthermore, the T_m value of outer primer (F3 and B3) were set lower than that of F2 and B3 in order to ensure that synthesis occurred earlier from the inner primer than from the outer primers. In addition, the outer primers were used at 1/4-1/10 the concentration of the inner primer.

The formation of stem-loop DNA from a dumb-bell structure is critical for LAMP cycling. Notomi et al. (2000) examined the effect of various sizes of loop between F2c (B2c) and Flc (Blc) on amplification efficiencies and found that a loop of 40 bases or longer gave the best results.

The efficiency of LAMP depends on the size of the target DNA because one rate limiting step for amplification in this method is strand displacement DNA synthesis tested various sizes of DNA and found that the best results could be obtained with 130-200 by DNAs. DNA of more than 500bp, including F2 and B2 (Notomi et al. 2000).

DNA polymerase is another critical factor for efficient amplification. The best amplification was obtained with *Bst* polymerase for less than 10^{-23} mole target DNA. Z Taq DNA ploymerase was less efficient under the current conditions, but might be useful when polymerase has a to be added before heat denaturation of target DNA, because it is thermostable (Notomi et al., 2000).

Chemicals destabilizing the DNA helix were found to markedly elevate amplification efficiencies in LAMP. The presence of 0.5-1.5 M betaine or L-proline, which reduces base stacking, stimulated not only the overall rate of the reaction, but also increased target selectivity with a significant reduction in amplification of irrelevant sequences (Notomi et al., 2000).

3.10.6 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).

3.10.7 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.

3.10.8 Primers for LAMP (Eiken, 2005)

Design of Primers

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

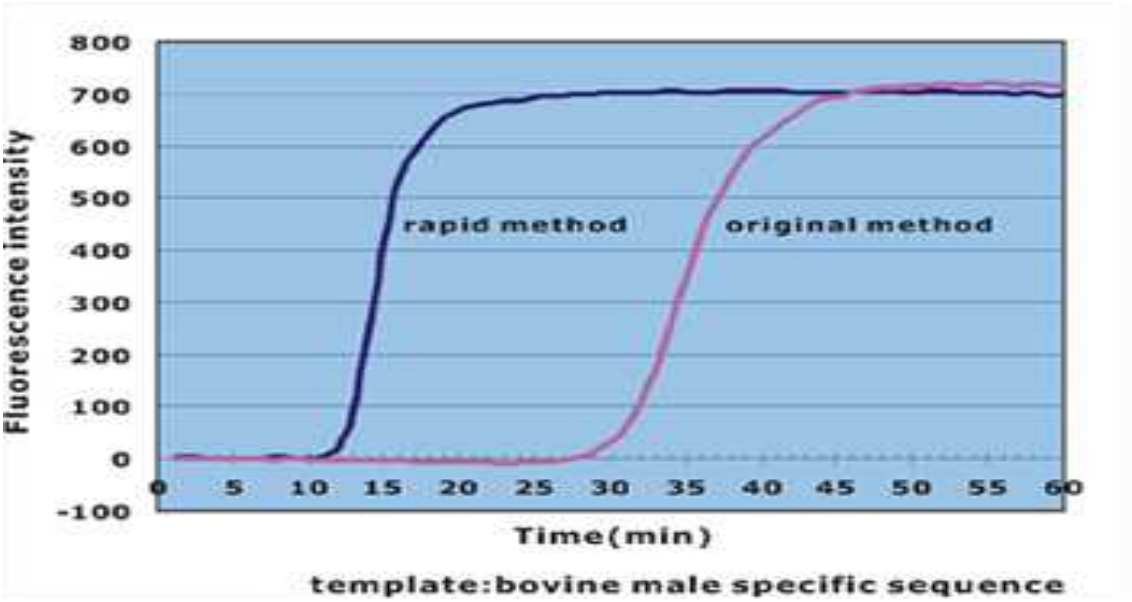
- If the restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products.

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.

Time Saving by Loop Primers (Eiken, 2005)

The investigation on how loop Primers affect amplification time (original method: no loop Primer; rapid method: with loop Primers) shows that the time required for amplification with loop primers is one-third to one-half of that without loop Primer. With the use of loop Primers, amplification can be achieved within 30 minutes.



Evaluation of primers

Iwamoto et al. (2003) performed LAMP with six primers: outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and loop primers (loop F and loop B) 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 (MGIT) or on a solid medium (Ogawa's medium). They recognized eight distinct regions on the target DNA. FIP consisted of a complementary sequence of F1 and a sense sequence of F2. BIP consisted of a sense sequence of B1 and a complementary sequence of B2. Species-specific primers MTB for *M. tuberculosis* complex, MAV for *M.avium*, and MIN for *M. intracellular* were designed to target the *gyr*B gene sequences and their specificities were validated on 24 mycobacterial species and 7 non mycobacterial species. The genus universal primers, Muniv, for the genus *Mycobacterium*, were specific for universally conserved mycobacterial 16S ribosomal DNA (rDNA) sequences. All of the sequence data for designing the primers were obtained from the Identification and Classification of Bacteria (ICB) *gyr*B database for *gyr*B, and from the ribosomal differentiation of medical microorganisms (RIDOM) data base for 16S rDNA (Appendix IV).

A successful LAMP reaction with species-specific primers produced many bands of different sizes. When the sample tube did not contain target DNA, no amplification was seen. To confirm that the amplification products had corresponding DNA structures, the amplified products were digested with restriction enzymes and the sizes of the fragments were analyzed by electrophoresis. The sizes of the fragments generated after digestion were in good agreement with the sizes predicted theoretically from the expected DNA structure. LAMP reaction mixture, which contained amplified fragments reactions, was simply judged by the naked eye with the addition of SYBR Green I (Iwamoto et al., 2003).

To evaluate the species specificities of the newly designed primers, Iwamoto et al. (2003) tested 35 mycobacterial strains and 7 nonmycobacterial species, which were grown on solid media. Significant amplification of the DNAs isolated from the targeted organisms was observed after 35-min incubation. In contrast, non-targeted strains were not amplified even after 60 min of incubation. Universal primers for the genus *Mycobacterium* were also used to confirm extraction of the proper DNAs. The primers amplified all of the mycobacteria after 35 min of incubation. Besides the mycobacteria, four actinomycetes were also amplified.

3.10.9 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 and Kearns 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 and Hermans 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; Hass et al.,1993 and Shawar et al.,1993). 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 (Cloud et al., 2002; Holberg et al., 1999 and Patel et al., 2000). 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 belogning 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 pputida 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).

Analysis of restriction fragment length polymorphisms (RFLP) allowed distinction of *M. tuberculosis*, M. *bovis, M. africanum, and M. mieran*. Niomann et al. (2000) exteneded these observations and proposed a diagnostic algorithm of *gyr*B-BFLP patterns to differentiate *M. tuberculosi/M. africanum II* from *M africanum I, M. bovis* subsp. bovis, *M. bovis* subsp caprae and *M. microti*.

Chimara et al. (2004) studied strains from six MTBC species-*M. tuberculosis, M. bovis* subsp. bovi, *M. bovis* BCG. *M. africamum, M. pinnipedii*, and *M. canetti* using *gvr*B-restriction fragment length polymorphism (*gyr*B-RFLP) analysis. A table was elaborated, based on observed restriction patterns and publisehd *gyr*B sequences. To evaluate applicability of *gyr*B-RFLP at Instituto Adolfo Lutz, Sao Paulo, Mycobacterial Reference Laboratory, 311 (MTBC clinical isolates, previously identified using traditional methods as *M. tuberculosis*, *M. bovis*, and *M. bovis BCG* were analyzed by *gyr*-RFLP analysis. All isolates were correctly identified by the molecular method but not distinction between *M. bovis* and *M. bovis* BCG was obtained.

3.10.10 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 strians 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 Enteroinvaive *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 h 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 x 10^2 copies, corresponding to 2-20 color changing units of *M. pneumoniae* in 1 h, 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.

Nagamine et al. (2001) performed LAMP reactions using genomic DNAs extracted from five HBV DNA-positive serum samples in which the initial copy number was unknown. When they used non-denatured DNA corresponding to 4 « l of serum as template, LAMP amplification was able to detect signals after 25-35 min in five individuals. This result revealed that the presence of HBV virus can be detected within 1 h from a non denatured sample. In separate experiments, however, LAMP was performed successfully without heat denaturation for template DNAs, such as DNA, pBluescript II, and M13 mp 18 vector DNA, and human genomic DNA (SRY gene on chromosome Y), including commercially available material. Some of the double-stranded DNA seemed to become single-stranded at high temperatures in the presence of high concentrations of betaine, a reagent that facilitates DNA strand separation

because it stabilizes DNA. Because there is no necessity for heat denaturation of the template DNAs, LAMP could be used more easily and rapidly in clinical medicine.

Nagamine et al. (2002 b) has developed a method to isolate single-stranded DNA fragments from LAMP products that are stem-loop DNAs with several inverted repeats of the target DNA. This method required the TspRI restriction enzyme, a primer hybridized to the 3' overhanging sequence at its cleavage site, and a DNA polymerase with strand displacement activity. The LAMP products were digested with TspRI and were then extended using the primer, producing the strand -specific DNA fragments. All processes, from LAMP reaction to primer extension, were carried out at the same temperature. So, the use of strand-specific DNA would be conducive for detection by hybridization technique such as DNA microarrays.

Mori et al. (2001) developed a method of detection of LAMP reaction by turbidity derived from magnesium pyrophosphate formation, yielding white precipitate of magnesium pyrophosphate in the reaction mixture. Judging the presence or absence of this white precipitate allowed easy distinction of whether nucleic acid was amplified by the LAMP method. Since an increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction was achieved by real-time measurement of turbidity.

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.

Nagamine et al. (2002) have developed a method that accelerates the LAMP reaction by using additional primers, termed loop primers. Loop primers hybridized to the stem loops, except for the loops that wee hybridized by the inner primer, and prime strand displacement DNA synthesis. Although both inner and loop primers reacted via the loops, they did so by different mechanisms. The LAMP method presented here used loop primers to achieve reaction times of less than half that of the original LAMP method. Since the total time of analysis including detection is less than 1 h, this new method should facilitate genetic analysis, including genetic diagnosis in the clinical laboratory.

Notami et al. (2000) developed LAMP in order to demonstrate the mechanism, the efficiency, and ease of use of LAMP. They chose M13mp18 DNA as a model target DNA, and prepared four primers that met the LAMP requirement. The LAMP produced many bands of different sizes from 300 bp to the loading well. Production of bands depended on the presence of inner primers, the template and DNA polymerase. When the products were analyzed by alkaline agarose gel electrophoresis, smeared DNA between bands and at the well was shifted to bands of < 10bp. To confirm the structure, the amplified products were digested with several restriction endonucleases and their sizes analyzed by electrophoresis. Similarly, southern blot results of the Pvu II and Bam HI digests perfectly agreed that the amplified DNA originated from target M 13 DNA.

Endo et al. (2004) detected the species-specific gp43 gene of *Paracoccidiolides brasiliensis* by LAMP in 22 clinical and seven armadillo-derived isolates. The amplified DNA appeared as a ladder with a specific bandings pattern. They were also able to obtain positive results from DNA extracted from a paraffin-embedded tissue sample of *paracoccidioidonycosis,* suggesting that this method may achieve clinical application in the near future.

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%.

Kaneko et al. (2005) used a LAMP assay for the detection of herpes simplex virus types I (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus (VZV). The virus specificities of primers were confirmed by using 50 HSV-1, 50 HSV-2, and 8 VZV strains. The assay was performed for 45 min at 65^0 C. The LAMP assay had a 10-fold high sensitivity than a PCR assay. An analysis of nucleotide sequence variations in the target and primer regions used for the LAMP assay indicated that 3 of 50 HSV-1 strains had single nucleotide polymorphisms. No HSV-2 or VZV strains had nucleotide polymorphisms. Regardless of the sequence variation, there were no differences in sensitivity with the HSV-1 specific LAMP assays. To evaluate the application of the LAMP assay for clinical diagnosis, they tested clinical samples from 40 genital herpes patients and 20 ocular herpes patients. With the LAMP assay, 41 samples with DNA extraction and 26 direct samples without DNA extraction were identified as positive for HSV-1 or HSV-2, although 37 samples with DNA extraction and just one without DNA extraction were positive by a PCR assay. Thus, the LAMP assay was less influenced than the PCR assay by the presence of inhibitory substances in clinical samples. These observations indicate that the LAMP assay is very useful for the diagnosis of HSV-1, HSV-2, and VZV infections.

3.11 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.

Thai et al. (2004) reported the development and evaluation of a one-step singe-tube accelerated real-time quantitative reverse transcription RT-LAMP assay for rapid detection of the severe acute respiratory syndrome coronavirus (SARS-CoV) replicase gene. A total of 49 samples (15 throat washes, 13 throat swabs, and 21 combined throat and nasal swabs) were evaluated and compared to conventional RT-PCR. The RT-LAMP assay demonstrated 100-fold-greater sensitivity, with a detection limit of 0.01 PFU. The sensitivity and specificity of RT-LAMP assay for detecting viral RNA in clinical specimens with regard to RT-PCR were 100 and 85%, respectively. The specificity of the RT-LAMP assay was further validated by restriction analysis as well as nucleotide sequencing of the amplified product. The concentration of virus in most of the clinical samples was 0.1 PFU (0.1 to 102 PFU), as determined from the standard curve of SARS RT-LAMP and based on the time of positivity. Thus, the RT-LAMP assay reported here has the advantages of rapid amplification, simple operation, and easy detection and will be useful for rapid and reliable clinical diagnosis of SARS-CoV in developing countries.

Parida et al. (2004) developed a one-step, single tube, RT-LAMP assay for detecting the envelope gene of West Nile (WN) virus. When the sensitivity of the RT-LAMP assay was compared to that of conventional RT-PCR, it was found that the RT-LAMP assay demonstrated 10-fold higher sensitivity compared to RT-PCR, with a detection limit of 0.1 PFU of virus. By using real-time monitoring, 10^4 PFU of virus could be detected i as little as 17 min. The specificity of the RT-LAMP assay was validated by the absence of any cross-reaction with other, closely related, members of the Flavivirus group, followed by restriction digestion and nucleotide sequencing of the amplified product. These results indicate that the RT-LAMP assay is extremely rapid, cost-effective, highly sensitive, and specific and has potential usefulness for rapid, comprehensive WN virus surveillance along with virus isolation and / or serology.

Parida et al. (2005) reported the development and validation of a one-step, real-time and quantitative dengue virus serotype-specific RT-LAMP assay targeting the 3' noncoding region for the rapid detection and differentiation of dengue virus serotypes. The evaluation of the RT-LAMP assay use for clinical diagnosis with a limited number of patient serum samples, confirmed to be infected with each serotype, revealed a higher sensitivity by picking up 100% samples as positive, whereas 87% and 81% of the samples were positive by reverse transcription-PCR and virus isolation, respectively. The sensitivity and specificity of the RT-LAMP assay for the detection of viral RNA in patient's serum samples with reference to virus isolation were 100% and 93%, respectively. The optimal assay conditions with zero background and no cross-reaction with other closely related members of the Flavivirus family as well as within the four serotypes of dengue virus was established. None of the serum samples from healthy individuals screened in this study showed any cross-reaction with the four-dengue virus serotypes-specific RT-LAMP assay primers. These findings demonstrated that RT-LAMP assay has the potential clinical application for detection and differentiation of dengue virus serotypes, especially in developing countries.

3.12 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 (Appendix VI). Sensitivity and specificity, together with 'predictive accuracy' are inherent properties of a screening test (Park, 2002).

It is in general accepted that the actual value of diagnostic tests, that is their reliability for distinguishing between persons who have disease and those who have not, depends on two characteristics:

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.

Sensitivity and specificity are attributes proper to each diagnostic method. However, when these methods are used in the field, the certainty of the results is affected by the frequency of the phenomenon being measured or prevalence. It is easy to understand that, if the phenomenon is trying to measure is quite frequent, recognizing it becomes easy; on the other hand, if the phenomenon is infrequent, one runs the risk of not recognizing it when one sees it (Maher et al., 1997).

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).

In situation of high TB-prevalence, smear microscopy for TB suspect will be found positive with fairly high frequency. The positive predictive value (PPV) of the positive results will be high. The PPV is the probability of the disease being present, among those with positive diagnostic test results (Maher et al., 1997).

To the extent the prevalence decreases, the frequency of finding smear-positive cases will also decrease, and the relative weight of false positives will become greater. The PPV of smear microscopy will decrease as prevalence drops. The Negative predictive value (NPV) is the probability that the disease was absent, among those whose diagnostic test results were negative (Maher et al., 1997).

1. 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 thy do not have disease. It amounts to giving them a "false reassurance". The patient with a "false-negative" test result might ignore the development of signs and symptoms and may postpone the treatment. This could be detrimental if the disease in question is a serious one and the screening test is unlikely to be repeated within a short period of time. 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. Material and Methods

4.1 Material

A complete list of bacteriological media, reagents, chemicals, equipments, glass wares and miscellaneous materials used in the study are given in appendix I. Some of the reagents and chemicals required for LAMP are as below.

Primers

F3: 5' CTGGCTCAGGACGAACG 3'

FIP: 5' CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT 3'

FL: 5' GTTCGCCACTCGAGTATCTCCG 3'

BL: 5' GAAACTGGGTCTAATACCGG 3'

BIP: 5' TCGGGATAAGCCTGGACCACAAGACATGCATCCCCT 3'

B3: 5' GCTCATCCCACACCGC 3'

LAMP buffer - Tris-HCl (pH 8.8), KCl, NH_4SO_4, and 0.1% Triton X-100.

SYBER Green I – Fluorescence dye

Beatain - N,N,N-trimethylglycine

Deoxyribonucleoside triphosphate (dNTPs)- GTP, ATP, CTP and TTP

4.2 Methods

4.2.1 Study Site

This study was carried out from October 2005 to November 2006 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 Sputum Specimens

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 129 sputum specimens from 43 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 61 specimens from 61 patients were collected.

All collected sputum specimens were processed for microscopy and some for culture and LAMP. Only 69 specimens from study group A and 61 specimens from study group B were further examined by culture and LAMP.

4.2.3 Sample Collection

During sample collection, patients were instructed to take a deep breath and coughed deeply and vigorously. Patients were instructed to cover their mouths carefully while coughing and not to use oral antiseptics during the period of sample collection. Saliva and nasal secretions were not accepted (Forbes et al., 2000). Similarly sputum containing food particles, residues and other extraneous matter were also rejected.

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 then third on the spot (Maher et al.,1997). Only one sample was taken from the follow-up patients. All the samples were collected in leak proof, wide mouth, and transparent, sterile and stopper plastic container.

4.2.4 Sample Processing

Before sample processing, the entire sample was examined for the visual appearance to have a desired sample. After the visual examination of the sample, all the samples were further processed.

4.2.4.1 Digestion, Decontamination and Concentration (Figure 3)

Digestion, Decontamination and Concentration were done as per standard methodology (An et al., 1995). Briefly,

1) 1-5 ml of sample from container was transferred to the 50 ml centrifuge tube with a screw cap.

2) Fresh digestant was prepared by breaking ampoule of N-acetyl L-cystein (NALC) in bbl MycoPrep NALC-NaOH Solution bottle.

1) MycoPrep NaOH-NALC sodium citrate solution was added in a volume equal to the quantity of specimen. Tighten the cap.
2) Vertex lightly or hand mix for about 15-30 seconds. The tube was then inverted to expose the whole solution with NALC-NaOH solution.
3) Kept at room temperature, with gentle continuous shaking/rotation for 15 minute. After completion of shaking, it was stand at room temperature for 5 minutes to allow for settling of aerosols.
4) Specimen volume was made up to 50 ml with the addition of sterile MycoPrep phosphate buffer (Appendix III), ensuring that there is no cross-contamination. Mixed well.
5) The specimen was centrifuged at 3000 X g for 20 minute at 4-16^{o}C.
6) After centrifugation, the tube was left for 5 minutes to allow aerosols to settle.
7) In the class II biosafety cabinet, the supernatant was carefulluy decant into a suitable container containing a mycobactericidal disinfectant.
8) Using a sterile pipette, pellet was resuspended with 1 ml sterile phosphate buffer and lightly vortex or mixed with the pipette.
9) The resuspended pellet was then used for smears, LJ culture and DNA extraction.

4.2.4.2 Sputum Microscopy

Sputum Microscopy was performed following standard protocol (WHO 1998a). Briefly,

An appropriate amount of the sputum sample was taken from the container with the sterile cotton swab and transferred to the clean; grease free slide. The specimen was spread on the slide to the size 2x1cm and made it thin enough to be able to read through it. Then smear was allowed to air dry for 15 minutes without heating. Then, the smear was heat fixed placing the slide over the Bunsen burner three to four times with the smear uppermost and allowed to cool before staining. These overall processes were carried out inside the class II cabinet only.

Microscopic Examination of Smears by Flurochrome (FL) method.

1. The heat fixed slides were placed on a staining rack, with smears facing up.
2. The Rhodamine-auramine solution was poured on the slide to cover the entire smear and allowed to stand for 15 min.
3. After 15 minute the slides were washed with tap water and drained.
4. The slides were then decolorized 20% sulphuric acid and leave for 2 minutes.
5. The slides were gently washed with water again and drained.
6. The slides were covered with counter stain (e.g., Potassium permanganate) solution and left for 2 minutes.
7. The slides were gently washed with water and drained.
8. Then after, the slides were air- dried and the whole smear was examined under 10X and 40Xusing a UV light microscope for acid-fast bacilli.

The organisms appeared as yellowish rods against a dark background.

4.2.4.3 Sputum Culture

Sputum samples, which were acceptable as per IUATLD/ATS criteria, were further processed for culture (WHO, 1998b).

For culture, using the pipette used for adding phosphate buffer to the pellet,2-3 drops (0.1-0.2 ml) of concentrated and decontaminated 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. Examined for growth twice weekly for 4 weeks and then once weekly for up to 8 weeks. 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 in appendix IX.

4.2.4.4 Loop-Mediated Isothermal Amplification (LAMP)

Loop-Mediated Isothermal Amplification (LAMP) was performed with decontaminated sample for culture, following the protocol previously standardized by Iwamoto in Japan (Iwamoto et al., 2003).

4.2.4.4.1 DNA Extraction

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

1. About 250 ml of the concentrated and decontaminated material was transfered to the ependroff tube.
2. Thus collected sample was treated at 95^{0}c for 10 min in hot water bath.
3. Then repeat 3 times freezing and thawing (freezing at -20^{0}c for 20 min and thawing at room temperature for 20 min.).

Thus prepared DNA was used for PCR.

4.2.4.4.2 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. Sequence of Primers used to diagnose Mycobacterium tuberculosis complex are as follow.

Primers

F3: 5' CTGGCTCAGGACGAACG 3'

FIP: 5' CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT 3'

FL: 5' GTTCGCCACTCGAGTATCTCCG 3'

BL: 5' GAAACTGGGTCTAATACCGG 3'

BIP: 5' TCGGGATAAGCCTGGACCACAAGACATGCATCCCCT 3'

B3: 5' GCTCATCCCACACCGC 3'

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 1/10-diluted original SYBR Green I for the direct detection of LAMP amplicons in the reaction tube with naked eye.

4.2.4.4.3 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 SYBER Green I, a fluorescent dye which stains DNA and in large amount of amplicons give distinguished color.

4.2.4.4.4 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. Large amount of amplicons when bind with SYBER Green I, it fluoresces green indicating target gene amplification

Details of Standard Operating Procedure (SOP

Figure 3

N-Acetyl-L-Cysteine-Sodium Hydroxide Decontamination Procedure

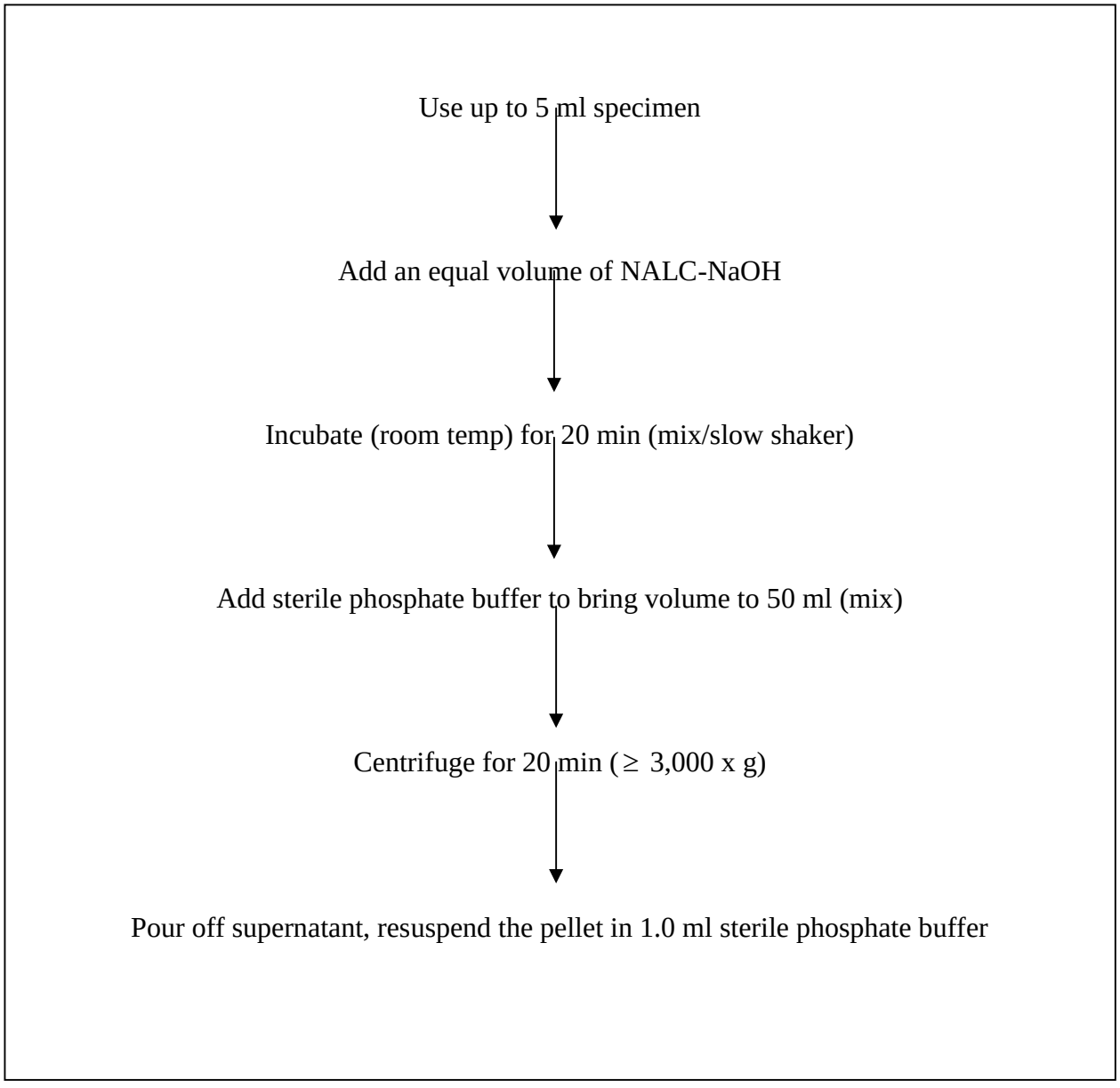
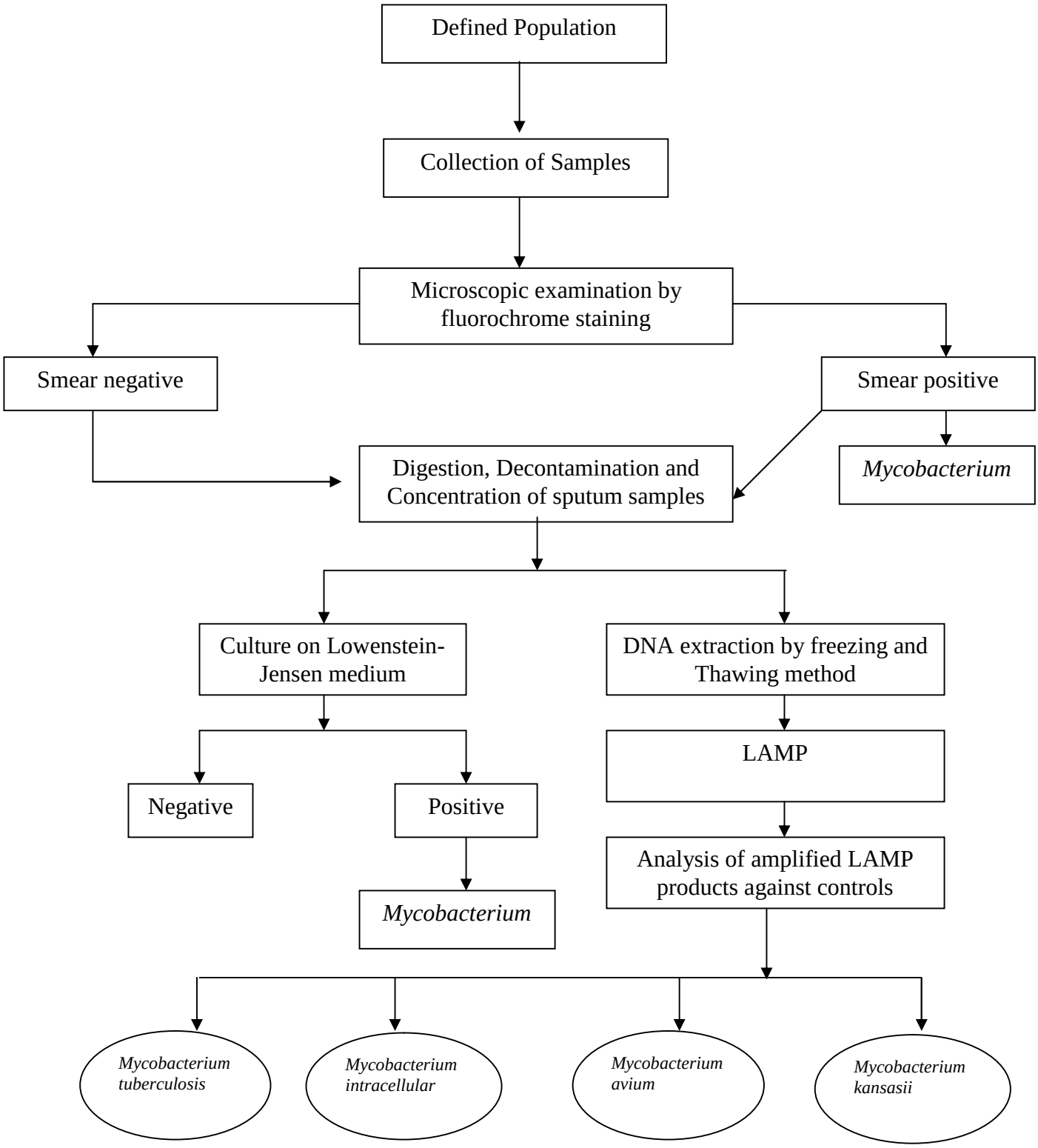


Figure 2: Flow Chart of Methodology



CHAPTER V

5. Results

A total of 190 sputum samples from two different study groups were collected in this study. Out of 190 samples, 129 were from new suspected tuberculosis patients and 61 from follow up patients. All the collected samples were examined by Microscopy. Among them, only 130 (69 from new suspected tuberculosis patients and 61 from follow up patients) sputum specimens were further examined by 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 Result

In this study, a total of 190 sputum samples were collected from two different study groups.

5.1.1 Study group A

This group includes 129 sputum samples from 43 **suspected tuberculosis patients** visiting National TB Referece Lab of GENETUP, kalimati..

5.1.1.1 Microscopy

From this group, all sputum specimens were examined by fluorochrome staining. Out of 129 (100%) sputum specimens, 55 (42.63%) were positive and remaining 74 (57.36%) were negative by fluorochrome staining.

5.1.1.2 Culture

From 129 sputum specimens, a total of 69 (100%) sputum specimens were examined by culture according to culture facility at the laboratory (Appendix XI). Among 69 sputum specimens, 40 (57.97%) were positive by culture while remaining 29 (42.03%) sputum specimens were negative by culture.

5.1.1.3 LAMP

Out of 69 (100%) sputum samples examined by LAMP for *M. tuberculosis*, *M. intracellular*, *M. avium* and *M. kansasii*, 44 (63.77%) were positive with LAMP and remaining 25 (36.23%) were negative.

Out of 44 LAMP positive cases 43 were positive with *M. tuberculosis* primer and one (90a) was positive with *M. intracellular primer*. *M. avium* and *M. kansasii* cases were not found in this study (Appendix XI).

Table 1: Comparative Results of 69 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	
34 (49.27%)	35 (50.73%)	40 (57.97%)	29 (42.03%)	43 (62.32%)	1 (1.45%)	-	-	44 (63.77%)	25 (36.23%)

Among 69 sputum specimens examined by Microscopy, culture and LAMP, 49.27% specimens were positive by fluorochrome stainng. Similarly, 57.97% sputum specimens were positive by culure on L-J media and 63.77% specimens were positive by LAMP.

5.1 .2 Study group B

This group includes 61 sputum specimens from 61 **follow up 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 flourochrome staining. Out of 61(100%) specimens, 20 (31.79%) were positive and 41 (67.21%) were negative by fluorochrome staining.

5.1.2.2 Culture

Out of 61 (100%) sputum specimens examined by culture on L-J medium, 31 (50.82%) were positive by culture while remaining 30 (49.18%) were negative.

5.1.2.3 LAMP

Out of 61 (100%) sputum specimens examined by LAMP for *M. tuberculosis, M. intracellular, M. avium* and *M. kansasii*, 34 (55.74%) were positive by LAMP and remaining 27 (44.26%) were negative by LAMP.

Out of 34 LAMP positive cases 33 were positive with *M. tuberculosis* primer while 1 (82a) was positive with *M. intracellular* primer.

Table 2: Comparative Results of 61 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	
20 32.78%)	41 (67.22%)	31 (50.82%)	30 (49.18%)	33 (54.1%)	1 (1.64%)	-	-	34 (55.74%)	27 (44.26%)

Among 61 sputum specimens examined by microscopy, culture and LAMP, 32.78% specimens were positive by microscopy. Whereas 50.82% specimens were positive by culture on L-J medium. Similarly 55.74% specimens were positive by LAMP.

5.1.3 Comparative results of total sputum specimens

A total of 130 (100%) sputum specimens (69 from study group A and 61 from study group B) were included in this study for comparative study of Microscopy, culture and LAMP. Among them, 54(41.54%) were microscopy positive. Similarly 71(54.62%) sputum specimens were positive by culture and 78(60%) sputum specimens were positive by LAMP.

Table 3: Comparative Result of Microscopy, Culture and LAMP from 130 Samples

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
54 54%)	76 (58.46%)	71 54.62%)	59 (45.38%)	76 (58.46%)	2 (1.54%)	-	-	78 (60%)	52 (40%)

Among the total 78(100%) LAMP positive cases, 76(97.44%) cases were *M. tuberculosis* where as 2(2.56%) cases were *M. intracellular*. *M. avium* and *M. kansasii* cases were not found from the samples included in this study.

5.1.4 Distribution of Total Culture Positive sputum.

Table 4: Age and Sex wise distribution of total culture positive cases.

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	7	9.86	0	0	7	9.86
21-30	19	26.76	7	9.86	26	36.62
31-40	13	18.31	6	8.45	18	26.76
41-50	9	12.67	2	2.82	11	15.49
51-60	1	1.41	0	0	1	1.41
Above 60	6	8.45	1	1.41	7	9.86
Total	55	77.46	16	22.54	69	100.00

Out of 130 sputum samples examined by culture, 71(n=100) samples were culture positive. Among them 55(77.46%) were male and 16(22.54%) cases were female. The highest numbers of culture positive cases were found in an age group 21-30(36.62%), followed by 31-40(26.76%), 41-50 (15.49%), 10-20 (9.86%) and above 60(9.86%).

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

Table 5: Age and Sex wise distribution of total *M. tuberculosis* cases.

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	8	10.52	0	0	8	10.52
21-30	22	28.95	7	9.21	29	38.17
31-40	13	17.10	6	7.89	19	24.99
41-50	12	15.79	1	1.32	13	17.11
51-60	1	1.32	0	0	1	1.32
Above 60	5	6.58	1	1.32	6	7.9
Total	61	80.26	15	19.74	74	100.00

Out of total 78 LAMP positive samples 76(n=100%) were *M. tuberculosis.* Among them, 80.26 %(n=61) were male and 19.19.74 %(n=15) were female in the age group between 10-79 years. Among them the highest number of cases was found in an age group 21-30(38.16%) followed by 31-40(24.99%), 41-50(17.11%), and 10-20 (10.52%) and above 60(7.9%). Lowest numbers of cases were found in the age group between 51-60 (1.32%).

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

Table 6: 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	1	50	0	0	1	50
Above 60	1	50	0	0	1	50
Total	2	100.00	0	0	2	100.00

Out of total 78 LAMP positive samples, 2 (n=100) were found *M. intracellular* positive. All (100%) cases were found in male and all were found in the elderly people i.e. above 50. One case was found from study group A and another case was found from study group B.

5.2 Quality Control

Before beginning of the study, we evaluate the primers for cross-reaction among *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii*.

Table 7: Evaluation of Primers

Primers used	*M. tuberculosis* control(TB_1)	*M.avium* control	*M.intracellular* control	*M. kansasii* control	No Template
MTB	Positive	Negative	Negative	Negative	Negative
MAV	Negative	Positive	Negative	Negative	Negative
MIN	Negative	Negative	Positive	Negative	Negative
MK	Negative	Negative	Negative	Positive	Negative

For this purpose we used five tubes each containing LAMP reagent and positive control of *M. tuberculosis, M. avium, M. intracellular,M. kansasii* and tube without DNA template respectively for evaluation of one primer. LAMP was performed by using only one primer at once and observed for species-specificity but no such cross-reaction was found.

5.3 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.

Out of 130 sputum samples subjected to culture and LAMP for the diagnosis of tuberculosis and other atypical mycobacteriosis, 67 samples were positive by both tests and 4 were positive only in culture, among them 48 were negative by both tests. While comparing the LAMP result with culture as gold standard, the sensitivity of LAMP was 94.36%, specificity was 81.36%, predictive value of positive test was 85.90%, predictive value of negative test was 92.31%, percentage of false positive was 5.63% and percentage of false negative was 18.64%.

Table 8: Comparison of LAMP with reference to culture

	Test and Results	Culture		Sensitivity	Specificity	PV+	PV-	False - ve	False +ve
		+ve	-ve						
LAMP	Positive	67	11	94.36%	81.36%	85.90%	92.31%	5.63%	18.64%
	Negative	4	48						

Where, PV +: predictive value of positive test, and PV- : predictive value of negative test.

Out of 130 samples subjected to culture and LAMP for the diagnosis of tuberculosis and other atypical mycobacteria, 67 samples were positive by both tests and 4 were positive only in culture, among them 48 were negative by both tests. While comparing

the LAMP result with culture as gold standard, the sensitivity of LAMP was 94.36%, specificity was 81.36%, predictive value of positive test was 85.90%, predictive value of negative test was 92.31%, percentage of false negative was 5.63% and percentage of false positive was 18.64%.

Table 9: Comparison of LAMP with reference to microscopy

LAMP	Test and Results	Microscopy		Sensitivity	Specificity	PV+	PV-	False - ve	False +ve
		+ve	-ve						
	Positive	53	25	98.14%	67.11%	67.94%	98.07%	1.85%	32.89%
	Negative	1	51						

Among 130 samples studied by microscopy and LAMP, 53 were positive by both tests whereas 25 samples were positive by LAMP but negative by microscopy. 1 sample was positive by microscopy but negative by LAMP. With reference to microscopy, the sensitivity and specificity of LAMP was 98.14%and 67.11% respectively.

CHAPTER VI

6 Discussion and Conclusion

6.1 Discussion

The rapid detection and identification of mycobacterial pathogens in sputum samples are necessary for the effective treatment of tuberculosis as well as pulmonary disease due to atypical mycobacteria. 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 were calculated to compare LAMP with cultural and microscopic methods of diagnosis. This is the first research in our country in which LAMP has been used for the diagnosis of *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii*.

The conventional methods used for the diagnosis of mycobacterial pulmonary diseases are clinical symptoms, chest X-ray, sputum smear microscopy, tuberculin skin test and in vitro culture methods for tuberculosis. But these methods have their own limitations. The rapid diagnosis of mycobacterial disease relies primarily on the detection of acid-fast bacteria by microscopy; however, the detection limit for microscopy is about 5,000-10,000 bacteria per ml and is nonspecific for species identification and differential diagnosis. Though cultural method can identify the infecting species, it is a time consuming procedure. A test that combines the rapidity of microscopy and the sensitivity of culture and that can identify the mycobacterial species would be great help to the clinician during the initial treatment of the patient.

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.

During the past decade, various nucleic acid amplification –based methods such as the PCR-based Roche Amplicor system , the rRNA amplification-based Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test system, ligase chain reaction, the Q-beta replicase amplified assay, the nucleic acid sequence-based amplification have been developed to address the need for rapid and sensitive diagnosis of *M. tuberculosis* and other mycobacterial infections. These methods require either precision instruments for the amplification or elaborate methods for detection of the amplified products, which are the major obstacles to wide use of these methods in relatively small scale clinical laboratories such as those in private clinics. In this regard, the LAMP- based assay developed in this study has the advantages of rapid reaction, simple operation and easy detection (Iwamoto et.al 2003).

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.

During the study period, a total of 130 sputum specimens were included to compare LAMP result with gold standard culture and Microscopy. Among the studied 130 sputum samples, 54 were fluorochrome staining positive. Out of 54 smear positive sampes, 51 showed growth 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 76 sputum samples were fluorochrome staining negative. Out of them, 20 samples were positive while culture on Lowenstein-Jensen medium. 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 even 100 organisms per ml can be detected by the solid medium. .

By culture, 71 sputum samples showed growth on the Lowenstein-Jensen medium. Upon testing by LAMP, 67 culture positive samples were positive indicating high sensitivity of LAMP. Remaining 4 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 Mycbactrial species(*M. tuberculosis*. *M .avium*,. *M. intracellular* amd *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 59 culture negative sputum samples 11 were positive by LAMP. From these 11 cultre negative samples, 8 were negative 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 78 LAMP positive samples, 76 were positive with *M. tuberculosis* primer and remaining 2 were positive with *M. intracellular* primer. None of the *M. avium* and *M. kansasii* cases were found during the overall study period. Tuberculosis infection was distributed among all age group with highest in 21-30 years but *M. intracellular* -pulmonary disease was found among elderly people. Among them one was found in new cases and another was found from was found in 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 the LAMP with culture as gold standard, the sensitivity of LAMP was 94.36 %, specificity was 81.36 %, predictive value of positive value was 85.90 %, predictive value of negative value was 92.31 %, percentage of false positive was 5.63% and percentage of false positive was 18.64 % respectively. 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 dreg, 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 PCR.

The possibilities of finding low specificity as compared with culture are supported by some other literatures. Vladimirskiy et al. (2003) used the PCR assay of MTB detection for to observe efficiency of specific chemotherapy of the patients with pulmonary tuberculosis. 18 from 21 observed patients analyzed for MTB excretion in sputum during 4-7 months every other 1.5 months by the culture on Lowenstein-Jensen solid medium and PCR assay exhibited a positive clinico-radiological changes and sputum conversion at the end of term of observation. In 4 patients with small processes without lung destruction MTB was not defined sputum by culture but it was found at 3 patients by PCR. These patients became to negative in 3 months of treatments. From 11 patients with infiltrative form with lung destruction MTB was finding at 9 cases by culture method in comparison with 11 patients in whom MTB was detected by PCR. The sputum conversion by culture these patients was occurred on the average in 1.8 months, in comparison with 4.7 months by PCR. 3 patients with fibro-cavernous tuberculosis of

lungs have exhibited a sputum conversion at 3 months by culture method and 5.7 months at average by PCR.

Paudel (2006) evaluated the LAMP result with gold standard culture. LAMP demonstrates high sensitivity and specificity.

With reference to Microscopy, the sensitivity of LAMP was 98.14%, indicating high accuracy rate of LAMP and specificity was 67.11%. 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 can not be detected by smear microscopy which may be positive by LAMP.

The finding of this study is comparable with and supported 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 50µl 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.

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 IS900fragment; 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/tuve 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.

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 2.2cfu/test tube using nine serotypes. The specificity was similar to that of a PCR assay, but the sensitivity of LAMP was greater.

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,* and *T. evansi*) and *T. congolense*. They showed that he sensitivity of the LAMP based method for detection to trypanosomes in vitro was up to 100 times higher than that of PCR based methods.

Kimura et al. (2005) evaluated the usefulness of LAMP for diagnosing central nervous system infection with herpes simplex virus (HSV). In his study, he 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 HSB infection of the central nervous system. When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 100%, the positive predictive value was 100% and the negative predictive value was 90%.

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*. 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 activities are the main burden for the effective control and management of tuberculosis. The conventional biochemical tests for identification of mycobacteial species are time-consuming because of the slow growth of mycobacteia on culture media. The LAMP-based assay can identify *M. tuberculosis* complex, *M. avium*, *M. intracellular* and *M. kansasii* from sputum samples directly within 1 hour. Hence the LAMP assay is more advantageous than all of the currently available DNA probe methods in its simple operation and rapid reaction.

6.2 Conclusion

In conclusion, the LAMP-based assay used in this study is novel nucleic acid amplification method that allows rapid and accurate identification of *M. tuberculosis*, *M. avium*, *M. intracellular* and *M. kansasii* in sputum samples. 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 recommendation

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 190 sputum samples belonging to two different categories were included in this study. Among the collected sputum samples, 129 were from 43 new suspected pulmonary tuberculosis patients and 61 were from 61 follow up patients.

All were examined by microscopy and 75 sputum samples were positive by fluorochrome staining while remaining 115 were negative.Among the total 190 sputum specimens collected, only 130 (69 from study group A and 61 from study group B) sputum specimens were further examined by culture and LAMP. These specimens were subjected to culture according to culture facility at GENETUP hospital.

While comparing the result of 130 sputum specimens with microscopy, culture and LAMP, 50(38.46%) samples were positive by all three diagnostic methods. On the other hand 48(36.92%) samples were negative by all. 1(0.77%) LAMP negative samples were positive by culture and microscopy. 3(2.31%) culture negative samples were positive by LAMP and microscopy. Similarly 3(2.31%) culture positive samples were negative by both LAMP and microscopy. 8 (6.16%) samples were LAMP positive even though they were negative by culture and microscopy.17 (13.07%) samples were positive by LAMP and culture but negative by microscopy.

Out of 78(100%) total LAMP positive cases, 76 (97.44%) were positive by *M. tuberculosis* primer and remaining 2(2.56%) were positive by *M. intracellular* primer. *M. avium* and *M. kansasii* cases were not found from the total samples included in this research.

When comparing the LAMP with culture as a gold standard the sensitivity of LAMP was 94.36%, specificity was 81.36%, predictive value of positive test was 85.90%,

predictive value of negative test 92.31%, percentage of false negative was 5.63% and percentage of false positive value was 18.64% respectively, however, with reference to microscopy, the LAMP had sensitivity 98.14% and specificity 67.11%.

7.2 Recommendation

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

1) LAMP performed in this study can be used for put forward and distinguishing of *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii* in sputum samples

2) In this study few culture negative samples were positive by LAMP particularly among follow up patients, which indicates faster sputum conversion rate of culture than LAMP. So LAMP may be an alternate method for evaluation of the drug efficacy.

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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Photograph 1: Culture of Mycrobacteria on Lwenstein-jensen medium

Photograph 2: Laboratory performance at the Lab

Photograph 3: Visual judgment of LAMP
P: positive control, 48a: sample (positive), 42a: sample (positive), 28a: sample (negative), 27a: sample (negative), N: negative control

Photograph 4: Laboratory performance of LAMP during the study period

APPENDICES

Appendix I: Materials

A) Bacteriological media

Lowenstein-jensen Medium (L-J Medium)

B) Reagents/ Chemicals

Absolute ethanol

Auramine-O

Acid-alcohol

Buffer

Bst DNA Polymerase

Betaine

dNTPs(dATP, dTTP, dCTP, dGTP)

Distilled water

Egg

Glycerol

Lysol

Magnesium Sulphate

Malachite green

Methylene Blue

N-acetyl-L-cysteine

primer

Sodium citrate

Sodium hydroxide

SYBR green I

C) Glasswares

Beaker

Capillary cuvette

Pipettes

Slides

Test tubes

Conical flask

Glass rod

Measuring cylinder

D) Equipments

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

Pipettes and tubes

Eppendrof tubes

Micropipette

Micropipette tips

PCR tubes

Miscellaneous

Bacteriological loop

Bunsen burner

Cotton Forceps

Gloves

Labeling stickers

Staining rack

Spit lamp

Soaps

Tube holder

Tissue paper

Appendix II: Bacteriology media

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

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

Ingredients

i) Mineral salt solution

Potassium dihydrogen phosphate,anhydrous[KH_2PO_4]	2.40gm
Magnesium sulphate [$MgSO_4$]	0.24gm
Magnesium citrate,quadrihydrate [$Mg(C_6H_5O_7)_2.4H_2O$]	0.60gm
Asparagine	3.60gm
Glycerol	12ml
Distilled water	600ml

Dissolve these ingredients in the water by heating and through mixing. Boil the solution for 2 hours in a steamer. Sterilize in an autoclave at 121^{o}C and 15-lbs pressure for 20 minutes.

ii) Malachite green solution

Prepare malachite green solution (2gm/dl) in sterile condition. Placing the solution in Incubator at 37^{o}C for 2 hours will help to dissolve the dye.

iii) Egg solution

Prepare egg solution, esing fresh eggs (not more than 4 days old). Depending on the size of the eggs, 20-24 eggs are needed. Wash the eggs carefully with soap and warm water, using the brush. Rinse I running watr for 30minutes. Drainthe water. Place eggs in sterile tray. Cover with sterile paper and leave to dry until next day. (This can be done by cleaning shells of eggs with methylated spirith and then burning off the excess spirit.)

Then thoroughly wash hands with soap and warm water. Rinse in running water, then with spirit and dry. Crack the eggs with a sterile knife and empty contents into a sterile beaker and beat with a sterile eggbeater.

Combine these three solutions as follows:

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

Mix them and distribute in 5ml aliquots in sterile screw-capped tubes or bottles.

Lay tubes or bottles on their sides and inspissate at 80-85^{o}C for 50 minutes. Medium can be leave in the inspissator obernight and then inspissate 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
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	1gm
Distilled water	1000ml

2. Solutions for sample decontamination

A. N-acetyl L-cystein(NALC)-NaOH solution

Working solution: 4% NaOH and Sodium citrate

i) 4% NaOH	50ml
ii)0.1M Sodium citrate	50ml

Mix i and ii, and sterilize by autoclaving at 121^{o}C(15lbs pressure) for 15 minute.

Add and dissolve 0.5gm N-acetyl L-cystein (NALC) just before use.

B. Phosphate buffer (PBS, pH 6.8,0.067M)

Dissolve 9.47gm of anhudrous disodium phosphate (Na_2HPO_4) in 1000ml (1 lit.) distilled/ deionized water, using a volumetric flask.

Dissolve 9.07gm monopotassium phosphate (KH_2PO_4) in 1000ml distilled/deionized water, using a volumetric flask.

Mix equal quantities of the two soulutions.Check the pH. Adding more solution A will raise the pH; more solution B will lower the pH. The final pH should be 6.8.

Sterilize by autoclaving.

3. 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.

A. LAMP Buffer

B.$MgSO_4$

Betaine (N,N,N-trimethylglycine)

Deoxyribonucleoside triphosphate (dNTPs)

***Bst* DNA polymerase**.

Primer

Template DNA

SYBR Green I

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

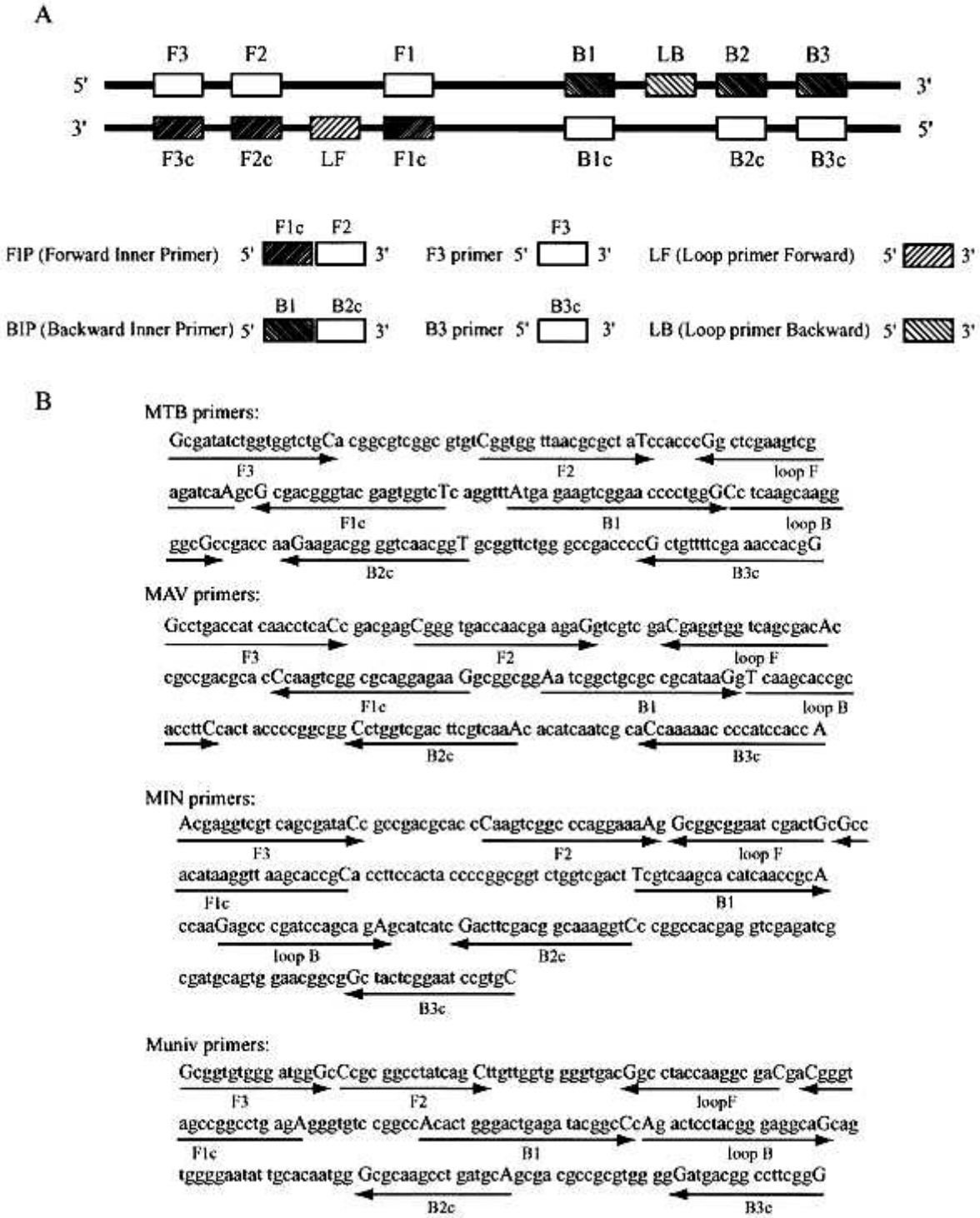
Molecular weight (µg/mol)	6149.0	Primer length	20
Millimolar extinction coeff. (OD/µmol)	217.4	Scale of synthesis	50n mol
Purity	Desalted	µg per OD	28.2
T_m(1M Na^+)	68	n moles per OD	4.6
T_m(50mM Na^+)	47	OD's	12.50
%GC	50	µg's	353.55
Coupling Eff.	99%	n moles	57.5

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)



Appendix V: Classification of Mycobacteria

Mycobacerial species-potential pathogens in humans

Complex name of closely related species	Species	Clinical significance
Tuberculosis complex	*M. tuberculosis* *M. bovis*	Both species are pathogenic and cause tuberculosis; they regularly exhibit susceptibility to anti-TB drugs; they are communicable
Leprosy group	*M. leprae*	Causes leprosy; cannot be cultivated in the laboratory media; can be grown in mouse footpad or in Armadillos, where the temperature is favourable for growth (2-5^{o}C below that of most mammals)
Runyon group		
I Photochromogens	*M. kansasii* *M. marinum* *M. simiae*	Usually pathogenic; susceptibility to isoniazid and rifampcin Usually pathogenic causes "Swimming pool granuloma"; only in skin lesions (never in sputum); susceptibility to streptomycin. Non pathogenic; resistant to anti-TB drugs
II Scrofulaceum complex (Scotochromogens)	*M. scrofulaceum* *M. szulgai*	Commonly non-pathogenic; resistance to anti-tuberculosis drugs. Newly recognized species; susceptible to ethambutol and rifampicin
III Battery-avium complex (Nonphotochromogens)	*M.avium* and/or *M. intracellular* *M. xenopi* *M. ulcerans*	Both species are usually pathogenic; most strains are resistance to anti-tuberculosis drugs. Commonly nonpahtogenic;susceptible to isoniazid and streptomycin Usulally pathogenic; found in superficial lesions
IV Rapid growers	*M. fortuitum* *M. chelonei*	Usually nonpathogenic; resistance to antituberculosis drugs. May be agents of abscesses in puncture wound; resistance to antituberculosis drugs.

Source: Pelczar; 1993

Appendix VI: Screening test result by diagnosis

Screening test results	Diagnosis		Total
	Diseased	Non 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 ut who don't have the disease (i.e. false positives)." c" includes those with negative test results but who have the disease (i.e., false negatives). Finally, those with negative rests 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) x100

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

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

Source: (Park, 2002)

Appendix VII: Statistical analysis of test

A) Comparison of LAMP with reference to culture

	Test and result	Culture		Total
		Positive	Negative	
LAMP	Positive	67 (a)	11 (b)	78 (a+b)
	Negative	4 (c)	48 (d)	52 (c+d)
	Total	71 (a+c)	59 (b+d)	130 (a+b+c+d)

Sensitivity= a/ (a+c) x100 = 64/71 x 100 = 94.36%

Specificity = d/ (b+d) x100 = 48/59 x 100 = 81.36%

Predictive value of positive test = a/ (a+b) x100 = 67/78 x 100 = 85.90%

Predictive value of negative test = d/ (c+d) x100 = 48/52 x 100 = 92.31%

Percentage of false negative = c/ (a+c) x100 = 4/71 x 100 = 5.63%

Percentage of false positive = b/ (b+d) x100 = 11/59 x 100 = 18.64%

Comparison of LAMP with reference to Micoscopy

	Test and result	Microscopy		Total
		Positive	Negative	
LAMP	Positive	53 (a)	25 (b)	78 (a+b)
	Negative	1 (c)	51 (d)	52 (c+d)
	Total	54 (a+c)	76 (b+d)	130 (a+b+c+d)

Sensitivity= a/ (a+c) x100 = 53/54 x 100 = 98.14%

Specificity = d/ (b+d) x100 = 51/76 x 100 = 67.11%

Appendix VIII: Treatment regimens for tuberculosis, MAC-PD and MK-PD

The essential anti-TB drugs, their ode of action and recommended dose (range in parenthesis).

Essential anti-TB drugs (Abreviation)	**Mode of action**	**Recommended dose (mg/kg) intermittent**		
		Daily	3x/week	2x/week[a]
Isoniazid (H)	Bactericidal	5(4-6)	10(8-12)	15(13-17)
Rifampicin (R)	Bactericidal	10(8-12)	10(8-12)	10(8-12)
Pyrizinamide (Z)	Bactericidal	25(20-30)	35(30-40)	50(40-60)
Streptomycin (S)	Bactericidal	15(12-18)	15(12-18)	15(12-18)
Ethambutol (E)	Bacteriostatic	15(15-20)	30(25-35)	45(40-50)
Thioacetazonone (T)	Bacteriostatic	2.5	Not applicable	

Source: Maher et al., 1997

[a] WHO doesn't generally recommend twice weekly regimes.

The current recommendation for treatment of pulmonary disease caused by *M. kansasii* in adults is the regimen given below.

Essential drugs	**Mode of action**	**Recommended dose(Mg/kg) Daily**
Isoniazid(H)	Bactericidal	300
Rifampin	Bactericidal	600
Ethambutol(E)	Bacteriostatic	25 for 2 month,then 15 daily
Clarithromycin		Altrnatives

Source: American journal of Respiratory and critical care Medicine, 1997

Initial therapy for adult HIV-negative patients with *M. avium* complex pulmonary disease.

Essential drugs	**Mode of action**	**Recommended dose**	
		Clarithromycin	**Azithromycin**
Clarithromycin or azithromycin	Bactericidal	500 mg 2x daily	250 mg/d or 500mg 3x/week
Rifamycin(Ricampin or Rifabutin)	Bactericidal	**Rifampin**	**Rifabutin**
		300 mg/d	600mg/d
Ethambutol(E)	Bacteriostatic	25mg for 2 month,then 15mg/d	

Source: American journal of Respiratory and critical care Medicine, 1997

Appendix IX: Grading of culture result

Reading	**Report**
No growth	Negative
1-19 colonies	Positive(Number of colonies)
20-100colonies	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 b

Appendix X

Standard Operating Procedure for LAMP

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
SYBER Green I (FD)	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.

Appendix XI: Results of total samples with microscopy, culture and LAMP

A) Results of 129 sputum samples (Study group A)

S.N.	Sample Code	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
1	2a	26	M	Neg	Neg	Neg	Neg	Neg	Neg
2	2b	26	M	Neg	-	-	-	-	-
3	2c	26	M	Neg	-	-	-	-	-
4	43a	52	M	Pos	Pos	Pos	Neg	Neg	Neg
5	3b	52	M	Pos	-	-	-	-	-
6	3c	52	M	Neg	-	-	-	-	-
7	4a	20	M	Neg	Neg	Neg	Neg	Neg	Neg
8	4b	20	M	Neg	-	-	-	-	-
9	4c	20	M	Neg	-	-	-	-	-
10	5a	33	F	Pos	Pos	Pos	Neg	Neg	Neg
11	5b	33	F	Pos	-	-	-	-	-
12	5c	33	F	Pos	-	-	-	-	-
13	6a	28	M	Pos	Pos	Pos	Neg	Neg	Neg
14	6b	28	M	Pos	-	-	-	-	-
15	6c	28	M	Pos	-	-	-	-	-
16	7a	50	M	Pos	Pos	Pos	Neg	Neg	Neg
17	7b	50	M	Pos	-	-	-	-	-
18	7c	50	M	Pos	-	-	-	-	-
19	8a	50	F	Neg	Neg	Neg	Neg	Neg	Neg
20	8b	50	F	Neg	Neg	Neg	Neg	Neg	Neg
21	8c	50	F	Neg	Neg	Neg	Neg	Neg	Neg
22	9a	35	M	Pos	Pos	Pos	Neg	Neg	Neg
23	9b	35	M	Pos	Pos	Pos	Neg	Neg	Neg
24	9c	35	M	Pos	-	-	-	-	-
25	11a	68	M	Neg	Neg	Neg	Neg	Neg	Neg
26	11b	68	M	Neg	-	-	-	-	-
27	11c	68	M	Neg	-	-	-	-	-
28	12a	39	M	Neg	Pos	Neg	Neg	Neg	Neg
29	12b	39	M	Neg	-	-	-	-	-
30	12c	39	M	Neg	-	-	-	-	-
31	13a	21	F	Neg	Pos	Pos	Neg	Neg	Neg
32	13b	21	F	Neg	Pos	Pos	Neg	Neg	Neg
33	13c	21	F	Neg	Pos	Pos	Neg	Neg	Neg
34	14a	53	M	Neg	Neg	Neg	Neg	Neg	Neg
35	14b	53	M	Neg	Neg	Neg	Neg	Neg	Neg
36	14c	53	M	Neg	-	-	-	-	-
37	15a	50	M	Neg	Neg	Neg	Neg	Neg	Neg
38	15b	50	M	Neg	-	-	-	-	-
39	15c	50	M	Neg	-	-	-	-	-
40	16a	80	M	Pos	Pos	Pos	Neg	Neg	Neg

41	16b	80	M	Pos	Pos	Pos	Neg	Neg	Neg
42	16c	80	M	Pos	-	-	-	-	-
43	17a	24	M	Pos	Pos	Pos	Neg	Neg	Neg
44	17b	24	M	Pos	Pos	Pos	Neg	Neg	Neg
45	17c	24	M	Pos	Pos	Pos	Neg	Neg	Neg
46	18a	30	M	Neg	Neg	Neg	Neg	Neg	Neg
47	18b	30	M	Neg	-	-	-	-	-
48	18c	30	M	Neg	-	-	-	-	-
49	20a	28	M	Pos	Neg	Pos	Neg	Neg	Neg
50	20b	28	M	Pos	Neg	Pos	Neg	Neg	Neg
51	20c	28	M	Pos	-	-	-	-	-
52	22a	32	F	Pos	Pos	Pos	Neg	Neg	Neg
53	22b	32	F	Pos	Pos	Pos	Neg	Neg	Neg
54	22c	32	F	Pos	Pos	Pos	Neg	Neg	Neg
55	37a	40	M	Neg	Neg	Neg	Neg	Neg	Neg
56	37b	40	M	Neg	-	-	-	-	-
57	37c	40	M	Neg	-	-	-	-	-
58	38a	52	F	Neg	Neg	Neg	Neg	Neg	Neg
59	38b	52	F	Neg	-	-	-	-	-
60	38c	52	F	Neg	-	-	-	-	-
61	39a	32	F	Neg	Neg	Neg	Neg	Neg	Neg
62	39b	32	F	Neg	-	-	-	-	-
63	39c	32	F	Neg	-	-	-	-	-
64	41a	33	M	Pos	Pos	Pos	Neg	Neg	Neg
65	41b	33	M	Pos	Pos	Pos	Neg	Neg	Neg
66	41c	33	M	Pos	Pos	Pos	Neg	Neg	Neg
67	45a	23	M	Pos	Pos	Pos	Neg	Neg	Neg
68	45b	23	M	Pos	Pos	Pos	Neg	Neg	Neg
69	45c	23	M	Pos	Pos	Pos	Neg	Neg	Neg
70	46a	44	M	Pos	Pos	Pos	Neg	Neg	Neg
71	46b	44	M	Pos	Pos	Pos	Neg	Neg	Neg
72	46c	44	M	Pos	-	-	-	-	-
73	47a	30	F	Pos	Pos	Pos	Neg	Neg	Neg
74	47b	30	F	Pos	Pos	Pos	Neg	Neg	Neg
75	47c	30	F	Pos	-	-	-	-	-
76	49a	76	F	Neg	Neg	Neg	Neg	Neg	Neg
77	49b	76	F	Neg	-	-	-	-	-
78	49c	76	F	Neg	-	-	-	-	-
79	55a	47	M	Neg	Neg	Neg	Neg	Neg	Neg
80	55b	47	M	Neg	Neg	Neg	Neg	Neg	Neg
81	55c	47	M	Neg	Neg	Neg	Neg	Neg	Neg
82	56a	41	M	Pos	Pos	Pos	Neg	Neg	Neg
83	56b	41	M	Pos	Pos	Pos	Neg	Neg	Neg
84	56c	41	M	Pos	Pos	Pos	Neg	Neg	Neg
85	59a	53	F	Neg	Neg	Neg	Neg	Neg	Neg
86	59b	53	F	Neg	-	-	-	-	-

87	59c	53	F	Neg	-	-	-	-	-
88	61a	21	M	Neg	Pos	Pos	Neg	Neg	Neg
89	61b	21	M	Neg	Pos	Pos	Neg	Neg	Neg
90	61c	21	M	Neg	Pos	Pos	Neg	Neg	Neg
91	62a	41	M	Neg	Neg	Pos	Neg	Neg	Neg
92	62b	41	M	Neg	-	-	-	-	-
93	62c	41	M	Neg	-	-	-	-	-
94	64a	31	F	Neg	Pos	Pos	Neg	Neg	Neg
95	64b	31	F	Neg	-	-	-	-	-
96	64c	31	F	Neg	-	-	-	-	-
97	65a	26	M	Neg	Neg	Neg	Neg	Neg	Neg
98	65b	26	M	Neg	-	-	-	-	-
99	65c	26	M	Neg	-	-	-	-	-
100	69a	56	F	Neg	Neg	Neg	Neg	Neg	Neg
101	69b	56	F	Neg	Neg	Neg	Neg	Neg	Neg
102	69c	56	F	Neg	Neg	Neg	Neg	Neg	Neg
103	78a	30	M	Neg	Neg	Neg	Neg	Neg	Neg
104	78b	30	M	Neg	-	-	-	-	-
105	78c	30	M	Neg	-	-	-	-	-
106	81a	32	M	Pos	Pos	Pos	Neg	Neg	Neg
107	81b	32	M	Pos	-	-	-	-	-
108	81c	32	M	Pos	-	-	-	-	-
109	87a	24	M	Neg	Neg	Pos	Neg	Neg	Neg
110	87b	24	M	Neg	-	-	-	-	-
111	87c	24	M	Neg	-	-	-	-	-
112	90a	52	M	Neg	Neg	Neg	Pos	Neg	Neg
113	90b	52	M	Neg	-	-	-	-	-
114	90c	52	M	Neg	-	-	-	-	-
115	93a	28	F	Neg	Neg	Neg	Neg	Neg	Neg
116	93b	28	F	Neg	-	-	-	-	-
117	93c	28	F	Neg	-	-	-	-	-
118	97a	62	M	Pos	Pos	Pos	Neg	Neg	Neg
119	97b	62	M	Pos	-	-	-	-	-
120	97c	62	M	Neg	-	-	-	-	-
121	98a	20	M	Pos	Pos	Pos	Neg	Neg	Neg
122	98b	20	M	Pos	-	-	-	-	-
123	98c	20	M	Pos	-	-	-	-	-
124	103a	22	M	Pos	Pos	Pos	Neg	Neg	Neg
125	103b	22	M	Pos	-	-	-	-	-
126	103c	22	M	Pos	-	-	-	-	-
127	104a	25	M	Pos	Pos	Pos	Neg	Neg	Neg
128	104b	25	M	Pos	-	-	-	-	-
129	104c	25	M	Pos	-	-	-	-	-

B) Results of 61 sputum samples (Study group B)

S.N.	Sample no	Age	Sex	Microscopy	Culture	LAMP Result			
						MTB	MIN	MAV	MK
1	1a	42	F	Neg	Neg	Neg	Neg	Neg	Neg
2	10a	42	M	Neg	Neg	Neg	Neg	Neg	Neg
3	19a	34	M	Neg	Pos	Pos	Neg	Neg	Neg
4	21a	16	M	Pos	Pos	Pos	Neg	Neg	Neg
5	23a	35	M	Neg	Neg	Neg	Neg	Neg	Neg
6	24a	25	M	Neg	Neg	Neg	Neg	Neg	Neg
7	25a	36	M	Neg	Neg	Neg	Neg	Neg	Neg
8	26a	28	F	Neg	Neg	Neg	Neg	Neg	Neg
9	27a	44	M	Neg	Neg	Neg	Neg	Neg	Neg
10	28a	25	F	Neg	Neg	Neg	Neg	Neg	Neg
11	29a	20	M	Neg	Neg	Pos	Neg	Neg	Neg
12	30a	60	M	Neg	Neg	Neg	Neg	Neg	Neg
13	31a	32	M	Neg	Neg	Neg	Neg	Neg	Neg
14	32a	21	M	Neg	Neg	Neg	Neg	Neg	Neg
15	33a	54	M	Neg	Neg	Neg	Neg	Neg	Neg
16	34a	28	M	Neg	Neg	Neg	Neg	Neg	Neg
17	35a	46	M	Neg	Neg	Neg	Neg	Neg	Neg
18	36a	48	M	Pos	Pos	Pos	Neg	Neg	Neg
19	40a	39	F	Neg	Pos	Pos	Neg	Neg	Neg
20	42a	38	M	Neg	Neg	Pos	Neg	Neg	Neg
21	43a	63	M	Neg	Pos	Pos	Neg	Neg	Neg
22	44a	32	M	Neg	Neg	Neg	Neg	Neg	Neg
23	48a	46	M	Neg	Neg	Pos	Neg	Neg	Neg
24	50a	32	M	Neg	Neg	Neg	Neg	Neg	Neg
25	51a	41	M	Pos	Pos	Pos	Neg	Neg	Neg
26	52a	43	M	Pos	Pos	Pos	Neg	Neg	Neg
27	53a	24	M	Pos	Pos	Pos	Neg	Neg	Neg
28	54a	24	M	Pos	Pos	Pos	Neg	Neg	Neg
29	57a	17	M	Neg	Pos	Pos	Neg	Neg	Neg
30	58a	28	M	Pos	Pos	Pos	Neg	Neg	Neg

31	60a	69	F	Neg	Pos	Pos	Neg	Neg	Neg
32	63a	30	F	Neg	Neg	Neg	Neg	Neg	Neg
33	66a	40	M	Pos	Pos	Pos	Neg	Neg	Neg
34	67a	40	M	Pos	Pos	Pos	Neg	Neg	Neg
35	68a	22	F	Pos	Neg	Pos	Neg	Neg	Neg
36	70a	37	F	Neg	Neg	Neg	Neg	Neg	Neg
37	71a	20	M	Pos	Pos	Pos	Neg	Neg	Neg
38	72a	29	M	Neg	Pos	Pos	Neg	Neg	Neg
39	73a	35	M	Pos	Pos	Pos	Neg	Neg	Neg
40	74a	42	F	Neg	Neg	Neg	Neg	Neg	Neg
41	75a	45	M	Neg	Neg	Pos	Neg	Neg	Neg
42	76a	29	F	Neg	Neg	Neg	Neg	Neg	Neg
43	77a	28	F	Pos	Pos	Pos	Neg	Neg	Neg
44	79a	35	M	Pos	Pos	Pos	Neg	Neg	Neg
45	80a	38	M	Neg	Pos	Pos	Neg	Neg	Neg
46	82a	65	M	Pos	Pos	Neg	Pos	Neg	Neg
47	83a	20	M	Neg	Pos	Neg	Neg	Neg	Neg
48	84a	20	M	Neg	Neg	Pos	Neg	Neg	Neg
49	85a	20	M	Neg	Neg	Neg	Neg	Neg	Neg
50	86a	20	M	Neg	Pos	Pos	Neg	Neg	Neg
51	88a	26	F	Neg	Pos	Neg	Neg	Neg	Neg
52	89a	39	M	Neg	Neg	Neg	Neg	Neg	Neg
53	91a	68	M	Neg	Neg	Neg	Neg	Neg	Neg
54	92a	29	M	Neg	Pos	Pos	Neg	Neg	Neg
55	94a	21	M	Neg	Pos	Pos	Neg	Neg	Neg
56	95a	45	F	Pos	Pos	Neg	Neg	Neg	Neg
57	96a	69	M	Pos	Pos	Pos	Neg	Neg	Neg
58	99a	24	M	Pos	Pos	Pos	Neg	Neg	Neg
59	100a	48	F	Neg	Neg	Neg	Neg	Neg	Neg
60	101a	45	F	Pos	Pos	Pos	Neg	Neg	Neg
61	102a	18	M	Pos	Pos	Pos	Neg	Neg	Neg