CHAPTER- I

1. INTRODUCTION

Tuberculosis (TB) is chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis* and characterized by the formation of granuloma in infected tissue as a result of cell-mediated immunity. Based on the site of involvement of infection and host response, TB is of different types; pulmonary tuberculosis (PTB), extra pulmonary tuberculosis and disseminated tuberculosis (Cole *et al*., 2005). Despite the availability of effective anti tuberculosis chemotherapy for over 50 years, it remains a major public health problem. Each year, there are two millions deaths occur due to tuberculosis, making TB the world's leading cause of mortality due to a single infectious agent (Mathema *et al*., 2006). It causes a great deal of ill health in the population of most low-income countries and it is the single most common cause of death in individuals aged fifteen to forty nine years (Enarson *et al*., 1996).

World Health Organization (WHO) declared TB as a global emergency. Eight million becomes sick every year and nearly three million cases per year occur in South-east Asia. The global epidemic is growing and becoming more dangerous. The breakdown in health services, the spread of human immunodeficiency virus (HIV) / acquired immune deficiency syndrome (AIDS) and the emergence of multidrug resistant TB are contributing to the worsening impact of these diseases. There were an estimated 8.8 million new cases in 2003; with an incidence rate of 141 per 100,000 populations (WHO, 2005). The global incidence rate of tuberculosis is growing at approximately 1.1% per year. Developing countries suffer the burnt of tuberculosis epidemic (CDC, 2007).

WHO estimates that between years 2000 and 2020, nearly 1 billion people will be newly infected. Approximately, 200 million people will get sick and 35 million will die from TB- if control is not further strengthenessd (Sohn *et al.,* 2003)

TB is one of the major public health problem in Nepal. About 45% of total population is infected with TB, out of which 60% are in adult age group. Every year, 40000 people develop active TB, of whom 20000 have infectious pulmonary diseases. Introduction of treatment by directly observed treatment short course (DOTS) have already reduced the number of deaths; however 5,000- 7,000 people continue to die every year from this disease (NTCP, 2004/2005).

Tools for the diagnosis of tuberculosis include clinical examination, tuberculin skin test (TST), chest radiograph, staining for acid-fast bacilli (AFB), culture for mycobacteria and more recently, nucleic acid amplification (NAA) assays. The TST was originally a test for active disease but it is unsuitable for that purpose because of its limited specificity. Diagnosis by means of radiographic examination is also unreliable because other chest diseases resemble TB on X-ray (Edwards and Edwards, 1960).

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. Staining procedure depends on the ability of mycobacteria to retain dye even when treated with mineral acid or an acid alcohol solution (Bloom, 1994; Salfinger and Pfyffer, 1994). Microscopy has a sensitivity and specificity low enough to be useful only as a presumptive screening test. However, because acid-fast smear results are available rapidly they are used to manage patient care and to make public health decision. Culture is considered as the most accurate test because of its high degree of sensitivity and specificity, however, is labor intensive and slow. Clinical laboratories hold cultures for 6 to 8 weeks to achieve the maximum sensitivity. 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 preformed (ATS- CDC, 2000; Kent and Kubica, 1985). The lack of early and accurate diagnosis is a critical obstacle to global TB control. The HIV epidemic has

further diminished the utility of routine microscopy and smear- negative TB has arisen as a particular problem in Sub-Saharan Africa (Watt *et al.*, 1996). The recent increase in tuberculosis cases and the emergence of MDR strains have demonstrated the need for more rapid and reliable methods of laboratory diagnosis. In such condition, NAA assay that amplify and detect specific nucleic acid sequences allow rapid, sensitive and specific detection of *M. tuberculosis* in sputum samples (CDC, 1992).

There are several NAA methods have been invented. Among them here we present the report of polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) for the detection of *M. tuberculosis* directly from the sputum samples. PCR is a method for oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. It is only simple and specific technique to amplify even a single molecule of DNA. The PCR product is amplified from the DNA template using a heat-stable DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase) and using an automated thermal cycler to put the reaction through 30 or more cycles of denaturing, annealing of primers and polymerization. After amplification by PCR, the products are separated by polyacrylamide gel electrophoresis and are directly visualized after staining with ethidium bromide under ultraviolet light (Sritharan and Sritharan, 2000)

In addition to PCR, LAMP is novel nucleic acid amplification method introduced by Notomi *et al*., (2000). LAMP has characteristics that may allow its use in less sophisticated settings. LAMP amplifies DNA with high efficiency under isothermal conditions using six sets of primers. The large amount of DNA generated and the high specificity of the reaction make it possible to detect amplification by visual inspection of fluorescence or instrument detection of the labeled probe. This allows the use of a closed tube system which minimizes the risk of workspace contamination with amplicon. It provides high amplification efficiency with DNA being amplified 10^9-10^{10} times within 15-60 minutes. The final amplification products are stem-looped DNAs

with several inverted repeats of the target and cauliflower like structures with multiple loops (Notami *et al*., 2000)

In this regards, the present study aims to evaluate the different diagnostic techniques for direct detection of *M. tuberculosis* in sputum samples. The sensitivity, specificity and applicability of microscopy, PCR and LAMP were evaluated by comparing to culture as 'gold standard'. It is expected that the outcomes of this study, will helps in rapid diagnosis, effective treatment and case management of TB in our country.

CHAPTER-II

2. OBJECTIVES

2.1 General objective

To compare the LAMP and PCR for the direct detection of *M. tuberculosis* in sputum

2.2 Specific objectives

a. To screen and identify *M. tuberculosis* rapidly in sputum samples using Fluorescence microscopy, Culture on Ogawa medium, LAMP and PCR

b. To evaluate the sensitivity and specificity of LAMP technique comparing with PCR, culture and microscopy

CHAPTER III

3. LITERATURE REVIEW

3.1 Disease

TB remains one of the primary infectious disease causes of morbidity and mortality worldwide, with over 95% of the cases and deaths occurring among adults in developing countries (WHO, 2005). It is a chronic bacterial infection caused primarily by *Mycobacterium tuberculosis* and less commonly by *M. africanum* or *M. bovis*, and characterized by the formation of granulomas in infected tissue and by cell mediated hypersensitivity (WHO, 1997). This disease has the potential to infect virtually every organ, most importantly the lungs due to dissemination via lympho-hematogenous route (Haas, 2000).

According to World Health Organization (WHO), about one third of the world's population are infected with *M. tuberculosis*, with about 10 million cases of active TB disease reported each year, leading to 3 million deaths annually (Dollin *et al.*, 1994; Mohan and Sharma, 2001). About 95% of TB cases and 98% of TB deaths are in developing countries and these deaths comprise 26% of all avoidable deaths. As 75% of infected people are less than 50 years of age (the most productive period in life), TB has the most devastating effect in the developing world (Sharma, 2001). The magnitude of the problem is enormous in view of the epidemic of HIV infection that has radically changed the epidemiology of TB. The close connection between HIV infection and TB was shown in the USA and in Africa where 15-70% of patients presenting with TB are HIV infected. Further, TB as a concurring problem in HIV infection is thought to be due to endogenous reactivation of the tubercle bacilli, as a result of the loss of immune control mechanisms in HIV infected individuals. This coupled to outbreaks of multiple drug resistant TB warrants stringent measures to combat this disease which is increasing at alarming rates (Sritharan and Sritharan, 2000).

3.2 Mycobacteria

Mycobacterium is a genus of *Actinobacteria*, given its own family the *Mycobacteriaceae*. The genus includes pathogens known to cause serious diseases in mammals, including tuberculosis and leprosy. The Latin prefix "*Myco*"- means both fungus and wax; its use here relates to the "waxy" compound in the cell wall. Organisms belonging to the genus *Mycobacterium* are thin, pleomorphic, straight or slightly curved rods between 0.2-0.6µm long. They are aerobic and nonmotile bacteria (except for the species *Mycobacterium marinum* which has been shown to be motile within macrophages) that are characteristically acid-alcohol fast. The high G+C content of the DNA of *Mycobacterium* species (61 to 70 mol % except for *Mycobacterium leprae*, [55%]) (Good and Shinnick, 1998) is within the range of those of the other mycolic acid containing genera including *Nocardia* (64 to 72 mol %), *Rhodococcus* (63 to 73 mol %), *Gordonia* (63 to 69 mol %), *Tsukamurella* (68 to 74 mol %), (Goodfellow, 1998).

All *Mycobacterium* species share a characteristic cell wall, thicker than in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acids / mycolates. The cell wall makes a substantial contribution to the hardness of this genus. An important character is their ability to resist decolorization by weak mineral acid after being stained by an arylmethane dye-acid fastness, but the genus is more accurately defined by the chemical structure (Minnikin, 1982).

Some species can be very difficult to culture. Sometimes taking over two years to develop in culture. Further, some species also have extremely long reproductive cycles. *M. leprae*, may take more tan 20 days to proceed through one division cycle (for comparison, some *Escherichia coli* strains take only 20 minutes), making laboratory culture a slow process (Cheesbrough, 1989).

There are 71 recognized species in the genus *Mycobacteria,* which produce spectrum of infection in human and animals (Good and Shinnick, 1998). Clinically *Mycobacteria*

are classified into two group; those associated with tuberculosis are collectively known as *M. tuberculosis complex (M. tuberculosis, M. africanum, M. bovis, M. microtii).* Other mycobacteria that may be associated with human disease are collectively known by different names such as atypical, anonymous, non-tuberculosis, tuberculoid, opportunistic and mycobacteria other than tuberculosis bacilli, usually abbreviated to MOTT (appendix XII). Many MOTTS are found in the environment but they can also colonize in man, as in the part of previously damaged respiratory tract and cause clinical infection (Watt *et al*., 1996).

Among the *Mycobacterium species, M. tuberculosis* is non-sporing, non capsulated, straight and slightly curved rod measuring 1-4×0.2-0.5µm (Cheesbrough, 1989). In sputum and other clinical specimens, they may occur singly or in small clumps, and in liquid cultures, they often grow as twisted rope like colonies termed serpentine cords (Greenwood *et al*., 2002). Tubercle bacilli are aerobes, slow growing with generation time of 12-24 hours and colonies usually appears in 2-3 weeks and may sometimes requires eight weeks of incubation at optimum temperature 37^{o}C and pH 6.4-7.0. If gram stain is performed, *M. tuberculosis* either stains very weakly gram positive, or not at all (Forbes *et al*., 2000). The tubercle bacilli are able to grow on a wide range of enriched culture media, but Lowenstein-Jenson (LJ) medium is widely used. The colonies of *M. tuberculosis* on LJ medium are dry, rough, and creamy or buff colored (Cheesbrough, 1989).

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. Single cough can produce 3,000-5,000 droplet nuclei. Patients who excrete 10,000 or more tubercle bacilli per ml of sputum are the main source of infection to others (Park, 2005). The AFB smear positive cases identifies

the most infectious patients, however both theoretical consideration and empirical observation indicates transmission does occur from smear negative (SN) patients (Groothuis and Yates, 1991). The threshold for detecting bacilli on light microscopy is about 10^4 bacilli /ml while the infecting dose of *M. tuberculosis* is estimated to be fewer than 10 organisms and report does show that patient with SN culture positive TB appears to be responsible for about 17% of TB transmission (Behr *et al*., 1999).

3.4 Risk of infection

TB can affect anyone and the risk to a susceptible individual of becoming infected depends on the density of tubercle bacilli in inhaled air and duration of exposure to that air (Riely and Nardell, 1999). The risk may be about 3 times higher (as with diabetes) to more than 100 times higher (as with HIV infection) for people who have these conditions than for those who do not. Some conditions appear to increase the risk that infection will progress to disease are;

- Alcoholic and intravenous drug users
- Babies and young children and old age
- Infection with HIV
- Recent TB infection (with in the past 2 years)
- Chest X-ray findings suggestive of previous TB
- Diabetes mellitus
- Silicosis
- Prolonged therapy with corticosteroids
- Immunosuppressive therapy
- Certain types of cancer (eg, leukemia, Hodgkin's disease, or cancer of the head and neck)
- Severe kidney diseases
- Certain intestinal conditions
- Low body weight (10% or more below ideal) (WHO, 2005)

Some drugs, including rheumatoid arthritis drugs that work by blocking tumor necrosis factor-alpha (an inflammation-causing cytokine), raise the risk of activating latent infection due to the importance of this cytokine in the immune defense against TB. The risk of transmission of infection from person with sputum smear negative PTB is low, and even lower from extra pulmonary TB (WHO, 2004).

3.5 Incubation period

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

3.6 TB infection versus TB disease

TB infection means that *M. tuberculosis* is in the body but the immune system is keeping the bacteria under control. The immune system does this by producing macrophages that surround the tubercle bacilli. The cells form a hard shell that keeps the bacilli contained and under control. Most people with TB infection have a positive reaction to the tuberculin skin test, people who have TB infection but not TB disease are not infectious, i.e., they cannot spread the infection to other people. These people usually have a normal chest X-ray. TB infection is not considered a case of TB (Cole *et al*., 2005).

Data from a variety of sources suggest that the lifetime risk of developing clinically evident tuberculosis after being infected is approximately 10%, with 90% like hood of latent infection. The risk of disease development is increased for those in high-risk groups, especially those with weak immune system. The chance of developing disease

is greatest shortly after infection and steadily lessens as time goes on (Harries *et al*., 1998).

Progression from TB infection to TB disease occurs when the TB bacilli overcome the immune system defenses and begins to multiply. In primary TB disease 1 to 5% of cases-this occurs soon after infection. However, in the majority of cases, a latent infection occurs that has no obvious symptoms. These dormant bacilli can produce tuberculosis in 2 to 23% of these latent cases often many years after infection (Parrish *et al*., 1998).

3.7 Pathogenesis

About 90% of those infected with *M. tuberculosis* have asymptomatic, latent TB infection, with only a 10% lifetime chance that a latent infection will progress to TB disease. However, if untreated, the death rate for these active TB cases is more than 50% (WHO, 2005).

Primary infection

The primary phase of *M. tuberculosis* infection begins with inhalation of the mycobacteria and ends with T-cell mediated immune responses that induce hypersensitivity to the organisms and controls 95 % of infection. Droplet nuclei that are inhaled into the lungs are so small that they avoid the mucobacillary defense of bronchi and lodge in the terminal of the lung (WHO, 2004). Most often in the periphery of one lung, inhaled *M. tuberculosis* is first phagocytosed by alveolar macrophage and transported by these cells to hilar lymph nodes. Naive macrophages are unable to kill the mycobacteria, which multiply, lyse the host cell, infect other macrophages, and sometimes disseminated through the blood to other parts of the lung and elsewhere in the body (Catran *et al*., 2000). In most of infected individuals, cell mediated immunity develops 2-8 weeks after infection. Activated T-lymphocyte and macrophages form granuloumas that limit further replication and spread of the organism (Schlunger and

Rom, 1998). The development of cell-mediated immunity against *M. tuberculosis* is associated with the development of a positive result in the tuberculin skin test (Mantoux test). Mycobacteria-activated T cells interact with macrophages in three ways: first CD4+ helper T cells secrete interferon-γ, which activates macrophages to kill intracellular mycobacteria through reactive nitrogen intermediates, including NO, NO2 and HNO_3. This is associated with the formation of epitheloid cell granulomas and clearance of the *Mycobacterium.* Second CD8+ suppressor T cell lyses macrophage infected with mycobacteria through a Fas-independent, granule development reaction and kill mycobacteria (Stenger, 1997). Third CD4-CD8-T cells lyse macrophages in a Fas dependent manner, without killing mycobacteria. Lysis of macrophages results in the formation of caseating granulomas. Direct toxicity of the mycobacteria to the macrophages may contribute to the necrotic caseous centers. Mycobacteria cannot grow in this acidic, extra cellular environment lacking in oxygen, and so the mycobacteria infection is controlled. The ultimate residual of the primary infection is a calcified scar in the lung parenchyma and in the hilar lymph node, together referred to the Ghon complex (Catran *et al.,* 2000).

Post primary infection

Post primary TB occurs following a latent period of month or years, after primary infection, approximately 10% of individuals with latent infection, progress to active disease, about half of these groups do so in the first 2 years after the initial infection and the remaining half develop active disease at less predictable times during the remaining years of life (Hornick, 1993). Post- primary TB can be caused either by reactivation of bacteria remaining from the initial infection or by failure to control a subsequent infection (Harries *et al.,* 1998). Granulomas of post primary tuberculosis most often occur in the apex of the lungs but may be widely disseminated in the lungs, kidney, meninges, marrow, and other organs. These granulomas, which fail to contain the spread of the mycobacterial infection are the major cause of tissue damage in tuberculosis and are reflection of delayed type hypersensitivity. Two special feature of

post primary tuberculosis are caseous necrosis and cavities, necrosis may cause rupture into blood vessels, spreading mycobacteria throughout the body, and break into airways, releasing infectious mycobacteria in aerosols (Catran *et al.*, 2000).

3.8 Genetic characters

Mycobacterial chromosomes were found to have molecular weight of (2.5-5.55) $\times 10^8$, with these of the major pathogen *M. tuberculosis* being at the lower end of the range. In addition to main chromosome, some strains contain one or more plasmid (Grange, 1990)

3.9 Treatment and control

Tuberculosis therapy generally consists of 6 to 9 months course of isoniazid, rifampicin, streptomycin, thioacetazone, pyrazinamide and ethambutol (Appendix XIII). Besides these main drugs, other includes cycloserine, ethionamide and capreomycin. These are three main properties of anti-TB drugs: bactericidal activity, sterilizing activity and the ability to prevent resistance (Maher *et al.*, 1997). Latent TB usually uses a single antibiotic, while active TB disease is best treated with combinations of several antibiotics, to reduce the risk of the bacteria developing *antibiotic resistance*. Drug - resistant TB is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs, MDR-TB is defined as resistance to the two most effective first line TB drugs: rifampicin and isoniazid extensively drug resistant TB (XDR-TB) is also resistant to three or more of the six classes of second-line drugs (O'Brien, 1994).

3.10 Diagnosis

The timely identification of infected person with *M. tuberculosis* and their rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measure for the control of the TB (Noordeen and Godal, 1998). Unfortunately,

delay in diagnosis is common; such delays can increase the risk of a poor outcome and lead to further transmission of tuberculosis, including the precipitation of out breaks in health care and institutional settings. Diagnosis of activated disease includes clinical suspicion, staining for acid-fast bacilli, culture for mycobacteria, and more recently, nucleic acid amplification assays (Foulds and O'Brien, 1998).

3.10.1 Clinical diagnosis

Clinically, pulmonary tuberculosis is chiefly present with persistent cough for three or more weeks, haemoptysis, shortness of breath and chest pain, loss of appetite and loss of weight, malaise and fatigue, night sweats and fever (Enarson *et al*., 2000). Symptoms of extra pulmonary tuberculosis depends on the organ involved (Enarson *et al*., 1996).

3.10.2 Radiological diagnosis

The radiographic appearance can also help in the detection of pulmonary mycobacteriosis but they do not allow etiological diagnosis. X-ray suggesting tuberculosis include upper-lobe infiltrates, cavitary infiltrates and hilar or paratracheal adenopathy. There is a radiologic difference between primary and secondary tuberculosis. Primary disease is usually characterized by single lesion in the middle or lower right lobe with enlargement of the draining lymph nodes. Endogenous reactivation is often accompanied by a single (cavitary) lesion in the apical region; with unremarkable lymph nodes and multiple secondary tubercles. Abnormalities on chest radiographs may be suggestive of, but are never diagnostic of TB. However, chest radiographs may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of disease (Mc Murray, 2001).

3.10.3 Tuberculin skin test

Infection in an asymptomatic individual can be diagnosed with the help of the intradermal PPD skin test. Intradermal introduction of PPD into a previously infected hypersensitive person results in the delayed (48-72 hrs) appearance of an indurated (raised, hard) reaction with or without erythema. It is impossible to distinguish between present and past infection based on a positive tuberculin test (McMurray, 2001). The tuberculin skin test also fails to detect a substantial proportion of persons co-infected with HIV and of person with advanced tuberculosis (ATS-CDC, 1981; Huebner *et al.*, 1993). Its sensitivity for active disease varies considerably from 65% to 94% (Lalvani *et al*., 2001).

3.10.4 Laboratory diagnosis

3.10.4.1 Microscopy

Microscopic examination of respiratory specimens for AFB plays a key role in the initial diagnosis of TB, monitoring of treatment, and determination of eligibility for release from isolation (Peterson *et al*., 1999). Sputum smear microscopy is the mainstay for diagnosis of TB. Microscopy is carried out by utilizing “Acid Fast" property of mycobacteria. The visualization of AFB in sputum or other clinical materials should be considered only presumptive evidence of TB, because stain does not specifically identify *M. tuberculosis* (Forbes *et al*., 1998). It has been observed that between 10^4-10^5 tubercle bacilli per ml of sputum are required for direct microscopy. Sputum specimens from patients with cavitary diseases are most often sputum smear positive (Katoach, 2004; Parekh and Kar, 2003).

There are several methods of determining the acid-fast nature of mycobacteria. In carbol-fuschin (Ziehl-Neelsen) procedure, acid fast organisms appear red against a blue background, while in the fluorochrome procedures, the acid fast organisms appear as fluorescent rods, yellow to orange (the color may vary with filter system used) against a

pale yellow or orange background (WHO, 1998a). Because fluorochrome-stained smears are viewed under high dry magnification rather than the oil immersion magnification required by fuschin-stained smear, they may be examined more rapidly and efficiently. Furthermore, with a fluorochrome dye, mycobacteria seen as staining bright against a dark background are easier to detect, hence smears examined by this method has been found to have a greater sensitivity than those stained with fuschin (Strumf *et al*., 1979). 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. Wherever possible, they should be obtained within twenty-four hours. 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).

However, direct microscopy of sputum, though rapid has low sensitivity (Habeenzu *et al*., 1980). In one study by Aber *et al*., (1980) found that in several African laboratories, the sensitivity of direct microscopy ranged from 8.8% to 46.4%. The sensitivity of direct sputum microscopy may be increased by concentration method. Kochhar (2002) found that out of 1484 samples, 9.3% positivity rate was observed on direct microscopy with the increase of yield by 30% after concentration.

3.10.4.2 Culture

The sensitivity of culture is excellent, ranging from 80% to 93% and specificity is quite high at 98%. Till date, culture is taken as “gold standard” method for diagnosis of tuberculosis, requiring only 10 to 100 microorganisms to detect *M. tuberculosis* (ATS-CDC, 2000). There are three types of culture media, which include egg-based media (Lowenstein-Jensen, Ogawa medium), and agar-based media (Middle brook 7H10 and 7H11), and liquid media (Middle brook 7H12 and other broths). Conventional culture of mycobacteria with solid media requires incubation for 3 to 6 weeks; use of broth-based

media can result in recovery of a positive culture 10 to 14 days sooner than with solid media. Broth-based media are also preferable because they are more sensitive than solid media, although neither type of medium recovers all isolates (Blumberg and Leonard, 2006)

Solid media are generally in practice as it allows visualization of colony morphology and pigmentation, which is useful in diagnosis for distinguishing the colonies of *M. tuberculosis* from those of some non tuberculous mycobacteria. Furthermore, DNA probes can be used for rapid identification of colonies of *M. tuberculosis complex*. These DNA probes provided species identification with in a few hours with nearly 100% accuracy if sufficient growth is tested. The L-J medium contains egg, glycerol, asparagines, mineral salts and malachite green dye (that inhibits certain contaminating bacteria). L-J medium is popular for isolating human strains of *M. tuberculosis* and most other mycobacteria (Salfinger *et al*., 1990). Ogawa's medium that is also in common use contains egg yolk instead of whole eggs (Grange, 1990). Most isolates appear between 3 and 6 weeks; a few isolates appear after 7 or 8 weeks of incubation. After 8 weeks of incubation, those showing no growth are reported as culture negative and discarded (Forbes *et al*., 2000). A radiometric detection system for mycobacteria growth developed by Middle brook *et al.*, (1977) has evolved into the BACTEC AFB system (Becton Dickinson diagnostic instruments, sparks, Md). Two types of BACTEC mycobacterial culture media are currently available. BACTEC 12B medium is broth medium that has been reported to yield more positive cultures from clinical specimens than other media. BACTEC 13A is used for bloods and bone marrow specimens (Rattan, 2001). It has been shown, that the nearly introduced mycobacteria growth indicator Tube (MIGIT), BACTEC 9000 MB, MB redox, MB / BACT and ESP II systems are suitable non-radiometric or fully automated alternatives to the radiometric BACTEC AFB system (Benjamin *et al*., 1998; Pfyffer *et al*., 1997; Tortoli *et al*., 1998)

3.10.5 Other techniques for diagnosis of tuberculosis

3.10.5.1 Immunological diagnostic methods

a. Antigen detection

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

b. Antibody detection

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

c. 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 (Kox, 1996).

d. **Tuberculostearic Acid (TBSA) Test**

One easily detected component of *M. tuberculosis* is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography (Brooks *et al*., 1987). The presence of tuberculostearic acid in cerebrospinal fluid is thought to be diagnostic for tuberculous meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis (Savic *et al*., 1992). However, an important concern with pulmonary specimens is that organisms other than *M. tuberculosis* may produce components that will generate a false positive signal (Bloom, 1994).

3.10.5.2 Nucleic acid amplification tests

Among the new approaches for the rapid diagnosis of TB, NAA methods are the most promising, but the technology is not applicable to control programmes in developing countries (Gebre *et al*., 1997). Two commercially available tests, Enhanced *M. tuberculosis* Direct Test (Gen-Probe MTD; San Diego, CA) and the Amplicor *M. tuberculosis* Test (Roche Diagnostic Systems) have been approved by the US Food and Drug Administration (FDA) for the detection of *M. tuberculosis* in respiratory specimens that are smear-positive for AFB and which have been increasingly used in industrialized setting (Hana and James, 2000; Ito, 2000). From the data reviewed by the FDA, the specificity (100%) and sensitivity (95% and 96% in the two studies) of these two tests in AFB smear-positive specimens were found to be comparable to the Accuprobe (Gen-Probe) for identification of *M. tuberculosis complex* in culture, with the advantage that the direct amplification test (DAT) results are available much sooner.

Molecular methods rely on extraction, amplification and detection of targeted nucleic acid of the conserved gene sequences of *M. tuberculosis*. These methods provide rapid detection, identification and characterization of the *M. tuberculosis* strains, different target sequence have used to confirm the diagnosis of TB (Niemann *et al*., 2000). Several molecular procedures are useful for diagnosis of mycobacterial diseases including strand displacement amplification (SDA), PCR, transcription-mediated

amplification (TMA), reporter phase systems, oligonucleotide ligation amplification and Q-beta replicase amplification. The first four of these amplification systems are the best developed of the system for mycobacteria (Bloom, 1994). More recently, LAMP has been proposed as rapid reaction, simple and easy detection system for the direct identification of Mycobacteria in clinical samples in less than 24 hours of specimen receipt (Iwamoto *et al*., 2003).

a. Strand displacement amplification (SDA)

SDA is an isothermal *in vitro* method of 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 oligonucleotides 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 primers to direct the DNA synthesis. When coupled with chemiluminescence-based hybridization detection system, the entire assay can be completed within 4h of obtaining processed specimen (Spargo *et al*., 1993). Species-specific SDA assays have been developed for the detection of *M. tuberculosis*, *M. avium*, and *M. kansasii*. An assay that detects many members of the *Mycobacterium* genus (a genus specific assay) has also been developed (Bloom, 1994).

b. Nucleic acid hybridization methods

Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes. The AccuPROBE SYSTEM uses a single-stranded DNA probe with a chemiluminescence label that is complementary to the ribosomal RNA of the target organism's ribosomal RNA to form a stable DNA: RNA hybrid. The selection reagent

allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA: RNA hybrids are measured in a GEN-PROBE luminometer (Kohne *et al*., 1984).

c. Transcription mediated amplification (TMA)

TMA, an isothermal target-based amplification system development by Gen-Probe Incorporation, has been combined with a homogenous detection method to detect *M. tuberculosis* in clinical specimen (Jonas *et al*., 1993). rRNA is amplified via TMA in which the rRNA target sequences are copies into transcription complex by using reverse transcriptase and then RNA polymerase is 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).

3.11 Polymerase chain reaction (PCR)

PCR, one of the most significant advances in DNA-based technology, is rapid, sensitive, inexpensive and simple means of producing relatively larger number of copies of DNA molecules from minute quantities of source DNA from viable or non-viable cell material even when the source DNA is of relatively poor quality (Sambrook et al., 1989). PCR has proved to be a most useful tool for the rapid diagnosis of infectious disease. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size (Sambrook and Russel, 2001). PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing, and primer extension (Mullis and Faloona, 1987). Descriptions of numerous PCR-based assays for the detection and identification of individual *Mycobacterium* species, such as *M. tuberculosis, M. leprae,* or *M. avium,* have been published recently. In general, the amplification process can be completed in 2 to 4 h of obtaining a processed specimens, and the detection assay can be completed in an additional 2 -24 h (Bloom, 1994).

3.11.1 The principle of PCR

PCR uses oligonucleotide primers bearing the complementary sequences that are unique to the target gene to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension by a thermostable enzyme DNA polymerase. The specificity of the amplification process lies in the choice of primers. It enables making millions of copies of a specific DNA sequence, which may be a gene, part of gene or simply a stretch of nucleotides with a known DNA sequence (Sritharan and Sritharan, 2000).

A specimen that may contain the sequence of interest is heated to denature double stranded DNA. The specific synthetic oligonucleotide primers bind to the unique DNA sequences of interest and heat stable DNA polymerase extends the primers to create a complete and complementary strand of DNA. This process is typically repeated 25-40 times within a period of 3-4 hours thereby creating millions of copies of the target DNA sequence. If the target sequence were not present in the sample, the primers have nothing to bind to and no amplification occurs. Thus the PCR offers potentially unmatched sensitivity and specificity. The diagnostic implications of such a technique is enormous. The amplified sequence can then be detected under ultra violet by gel electrophoresis after staining with ethidium bromide and/or by hybridization to radioactive or non-radioactive probes after transfer onto synthetic membranes (Sritharan and Sritharan, 2000).

3.11.2 Optimization of PCR

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR condition. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR reactions from potential DNA contamination. This usually involves spatial separation of PCR-setup areas from areas for analysis or

purification of PCR products, and thoroughly cleaning the work surface between reaction setups, primer-design techniques are important in improving PCR product field and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA (Sambrook *et al*., 1989).

Consequently, each new PCR application is likely to require optimization and it may be highly advantageous for a given application, especially when respective diagnostic or analytical procedures with optimal performance is necessary (William, 1989).

3.11.3 Components of PCR

PCR buffer

PCR buffer containing Tris- HCl, $Mgcl_2$ and KCl with pH 8.0 keeps the PCR mix at the proper pH so the PCR will take place further; magnesium is a co-factor for *Taq* polymerase and affects primer annealing, strand dissociation temperatures, specificity and primer-dimers.

Deoxyribonucleoside triphosphates (dNTPS)

The deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) provide both the energy and nucleosides for the synthesis of DNA. It is important to add equal amounts of each nucleotide to the PCR mix to prevent mismatches of bases.

Primers

A primer is a short segment of nucleotides, which is complementary to a section of the DNA, which is to be amplified in the PCR. Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule.

***Taq* polymerase**

Taq polymerase recombinant thermo stable DNA polymerase from the thermophilic bacterium, *Thermus aquaticus*, which adds the deoxynucleotides to the DNA template and retains activity after being heated to 95^{o}C. Heating and cooling steps, could be carried out on the same mixture without adding new enzyme. This allowed the procedure to be automated.

Template DNA

It is a piece of DNA present in sample, which will be amplified by the PCR. Successful amplification of the region of interest is depend upon the amount and quality of the template DNA.

Thermocycler

The PCR process is carried out in a thermal cycler. This is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. Most thermal cyclers have heated lids to prevent condensation on the inside of the reaction tube caps. Alternatively, a layer of oil may be placed on the reaction mixture to prevent evaporation (Pavlov *et al.*, 2004). It takes PCR mixtures through 20 to 50 cycles, producing large amounts of synthetic DNA for subsequent analysis (Bast *et al*., 2000).

3.11.4 The procedure of PCR

The procedure of PCR involves preparation of the sample, the PCR mix and the primers, followed by detection and analysis of the reaction products.

Sample preparation

The essential criterion for any DNA sample is that it contains at least one intact DNA strand encompassing the region to be amplified and that any impurities are sufficiently diluted so as not to inhibit the polymerization step if the PCR amplification, usually a 1:5 dilution of the sample with water is sufficient to dilute out any impurities, which may result the purifying protocol. Although any protocol is acceptable for PCR purposes, it s often best to use their use the fewest steps possible in DNA preparation in order to prevent accidental contamination with DNA (Bast *et al*., 2000)

PCR mix preparation

The PCR mix contains all of the components necessary to make new strands of DNA in the PCR process. The PCR mix reagents include buffer, deoxyribonucleotides, primers, *Taq* polymerase and template DNA.

Cycle parameters

The sequences of the primers are a major consideration in determining the temperature of the PCR amplification cycle. The annealing temperature must be calculated for each primer set. The higher temperature minimizes non-specific primer annealing, increasing the amount of specific product produced and reducing the amount of primer dimmer formation. The annealing temperature is usually about $5^{o}C$ less than the melting temperature (T_m) of the primer; however, this should be checked by performing the reaction at several temperatures.

The formula below can be used to calculate the melting temperature for oligonucleotides:

$$Tm = 81.5+16.6 \times (\log_{10} [Na^+]) +0.41\times (\%G+C)-675/n$$

Where, $[Na^+]$ is the molar salt concentration;

$[Ka^+] = [Na^+]$ and n = the number of bases in the oligonucleotide

(Promega, 1996).

Detection and analysis of the reaction product

The PCR product is a fragment or fragments of DNA of defined length. The simplest way to check for the presence of these fragments is to load a sample taken from the reaction product, along with appropriate DNA molecular weight markers; into an agarose gel. Staining with ethidium bromide facilitates the visualization of DNA bands on the gel under ultraviolet trans-illumination. Identification is done by comparing the bands with that of known markers (Eisenach *et al*., 1990).

3.11.5 Primers for PCR

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 /or other mycobacteria. Therefore, there are advantages to using genus specific primers in the amplification. The amplified product could then be identified with species-specific probes. Laboratories have used combination of *Mycobacterium*-specific primers for amplification, and the amplified product has been hybridized with specific probes or analyzed by restriction fragment length polymorphisms (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 several sets of primers have been constructed to detect *M. tuberculosis*. For example, primers have been developed to detect single-copy genetic elements encoding proteins such as the conserved mycobacterial 65-kDa heat shock protein or repetitive insertion sequences, such as *IS6110 (IS986)*, which are specific to and repeated 1 to 20 times in the chromosome of members of the *M. tuberculosis complex* (Kent *et al*., 1995).

3.11.6 Selection of primers

Mycobacterium tuberculosis complex have revealed an increasing number of targets for the study of historical cases of tuberculosis. PCR has been used to analyze various mycobacterial genes for diagnostic purposes, including 16S and 23S rRNA genes, genus-and species-specific fragments in the chromosome, genes coding for the 65-kDa heat shock protein and the 38-kDa protein B antigen and insertion sequences such as IS6110 (Eisenach *et al*., 1990). 16S rRNA has been reported to be a suitable target for use in PCR amplification assays for the detection of *Mycobacterium spp* in a variety of clinical samples and has frequently been used to identify various specific microorganisms because 16SrRNA genes show species-specific polymorphisms. However, because of extremely slow speed of the molecular evolution of 16S rRNA genes of closely related bacterial strains, such as those belonging to the *M. tuberculosis complex*, is either non existent or too small to differentiate between these species (Krischner *et al*., 1996).

MPB70 gene provides a highly specific and quantifiable target for molecular detection, as it is a single-copy gene found only in members of the tuberculosis complex.

MPB70 is a major secreted immunogenic protein component of tuberculosis. It is another protein present in *M. bovis* culture filtrate. High sensitivity and specificity have been reported in test where the antigen was used in an enzyme-linked immunosorbent

assay (EIA) and a fluorescence polarization assay. Hyecheong koo *et al.,* (2005) reported sensitivities 98.6% and 96.8% and specificities of 98.5% and 90.1% for the EIA with ESAT6-p and rMPB70, respectively.

Jerzy *et al.,* (1995) used multiplex PCR in a single tube in which DNAs of different sizes were amplified from a region of the MPB70 gene of *M. tuberculosis* (372 bp) and from a region of the 16S rRNA gene of members of the genus *Mycobacterium* (1,030 bp), *M. intracellulare* (850 bp) and *M. avium* (180 bp).

3.11.7 PCR for diagnosis of tuberculosis

A number of PCR based assay systems have been reported for diagnosis of *M. tuberculosis* in the laboratory conditions with specific isolates show very good performance with reference to specificity and sensitivity to a level of 1-10 bacilli per ml of the specimen (Sritharan and Sritharan, 2000).

Ziraweckij *et al.,* (1994) used PCR for the detection of pathogenic species *M. tuberculosis* in sputum sample. One pair of primers was synthesized for use in PCR with DNA-extracts and whole cell lysate from patient's sputum. Using PCR, identification value of pathogenic *M. tuberculosis* in sputum was increased from 44.1 % (Ziehl-Neelsen Staining) and 55.9% (Culturing) to 73.5%.

Kocagoz *et al., (*1993) used PCR for the detection of *M. tuberculosis* in 78 sputum samples tuberculosis was detected by PCR in all smear-and culture-positive and smear-negative, culture-positive cases. Additionally, PCR was capable of detecting four of nine cases, which were smear and culture negative but clinically suspected of tuberculosis.

Clarridge *et al*., (1993) investigated the use of DNA amplification by the PCR for detection of *M. tuberculosis* from 5000 clinical specimens. Two- thirds of each sample was processed for smear and culture by standard methods, and one-third was submitted

for DNA extraction, amplification of a 317-bp segment within the insertion element IS6110, and detection by agarose gel electrophoresis, hybridization, or both. DNA was prepared from 5,000 samples, with 623 samples being culture positive for acid-fast bacilli. Of 218 specimen that were identified as *M tuberculosis*, 181 (85%) were positive by PCR. In the *M. tuberculosis* culture positive for 136 of 145 (94%) and 45 of 73 (62%) of the fluorochrome smear-positive and smear-negative specimens, respectively. Of 948 specimens that were either culture positive for mycobacteria other than *M. tuberculosis* or culture negative, 937 specimens were negative by PCR and 11 (1%) specimens initially appeared to be false positive for *M. tuberculosis*. In comparison with culture, the sensitivity, specificity, and positive predictive value were 83.5%, 99.0% and 94.2%, respectively for PCR.

Noordhoek *et al.,* (1994) assessed the reliability, reproducibility and validity of PCR by a seven-laboratory blinded study for the detection of *M. tuberculosis* in clinical samples (200 sputum, saliva and water samples containing either known numbers of *M. bovis* BCG cells or no added organisms) as targeting the amplification of IS6110 using their own protocol for pretreatment, DNA extraction, and detection of the amplification product. In this study, several participating laboratories reported high level of false-positive PCR results, with ranging from 3 to 20% and with one extreme value of 77%. The levels of sensitivity also ranged widely among the different participants. A positive PCR result was reported for 2 to 90% of the samples with 10 mycobacteria.

Tansuphasiri (2000) comparatively evaluated three amplicon detection methods (agarose gel electrophoresis, Southern blot hybridization, dot blot hybridization) and found the detection limit 1pg DNA by gel electrophoresis for detection of IS6110-PCR products for the direct detection of *M. tuberculosis* in sputum samples and on comparison with culture, PCR showed overall sensitivity, specificity, positive predictive value, negative predictive value, and efficiency of 88.1%, 100%, 100%, 92.4%, and 95.1% respectively for agarose gel electrophoresis.

Gunisha *et al*., (2001) suggested that PCR using IS6110 to produce 123 bp fragment of DNA is 95.6% specific but only has a sensitivity of 30% to detect *M tuberculosis* in clinical specimens.

Sohn *et al*., (2003) used PCR for the detection of *M. tuberculosis* in 103 sputum samples, of these, 19 were positive by Z-N stain, 26 by PCR and 25 by culture. Four specimens of Z-N negative showed positive result in both culture and PCR. Two specimens of Z-N stain and culture positive were PCR negative. Five specimens showed positive result only with PCR. Two culture positive specimens gave negative results by both Z-N stain and PCR. Sensitivity, specificity, positive predictive value and negative predictive value of PCR were 84%, 93.25%, 80.8% and 94.9% respectively.

Condos *et al*., (1996) reported overall sensitivity and specificity of the PCR assay for a diagnosis of TB was 95% and 89% respectively. The PCR assay correctly identified 39 of 41 patients with proven PTB while 26 (63%) of whom were sputum-smear negative.

Beige *et al.*, (1995) evaluate the accuracy of the PCR assay; they performed a prospective study with 103 patients, comparing PCR results with culture results of samples obtained from a parallel culture assay as well as with subsequent culture results. Using two MTB- specific PCR primer systems, they found 48 of 49 tuberculosis (TB) patients to be PCR positive (PCR Sensitivity, 98%). Sixteen of 54 presumably non-TB patients showed amplifiable MTB DNA (Specificity, 70%).

3.12 Loop-mediated isothermal amplification (LAMP)

LAMP is novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. The characteristics of LAMP make it a promising platform for the molecular detection of tuberculosis (TB) in developing countries. This method does not require a denatured DNA template. The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize a total of six distinct sequences on the

target DNA (Eiken, 2005). 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 10^9 copies of target in less than an hour. 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 repeat 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 sequences with high selectively (Notomi *et al.*, 2000).

LAMP reaction appears to be limited only by amount of deoxyribonucleoside triphosphates 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 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 an infectious disease both in well-equipped laboratories and in field situations.

Furthermore, the addition of two more primers called loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primers (Nagamine *et al*., 2002). Eiken Chemical Company, in a joint development agreement with the

Foundation for Innovative New Diagnostics, has modified the technique and transformed it into a more convenient kit format (Catharina *et al*., 2007).

3.12.1 Characteristics of LAMP

LAMP has the following characteristics: (i) all reaction can be conducted under isothermal conditions ranging from 60^{o}C to 65^{o}C 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).

3.12.2 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,

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 (Fig.1).

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 (Fig.1).

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 (Fig.1).

Step4

A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand (Fig.1).

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 (Fig.1).

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 (Fig.1).

Step7

Double stranded DNA is produced through the processes described in Step (6) (Fig.1).

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 (Fig.1).

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 (Fig.1).

3.12.3 Components of LAMP

LAMP buffer

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

Deoxyribonucleoside triphosphate (dNTPs)

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

Bst DNA polymerase

Bst DNA polymerase 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 target 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 amplification of the region of interest is dependent upon the amount and quality of the template 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 (Iwamoto *et al*., 2003).

3.12.4 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 hour 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).

3.12.5 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^oC and 65^oC, 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 500 bp amplified, but very poorly. Therefore, the size of target DNA should be set to less than 300 bp, including F2 and B2.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 polymerase was less efficient under the current conditions, but might be useful when polymerase has 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).

3.12.6 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).

3.12.7 LAMP for a RNA target

LAMP is also applicable to RNA upon use of reverse transcriptase (RTase) together with DNA polymerase. The amplification depends on both RTase and *Bst* polymerase (Notomi *et al*., 2000).

3.12.8 Primers for LAMP

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 the 5' 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. (Eiken, 2005).

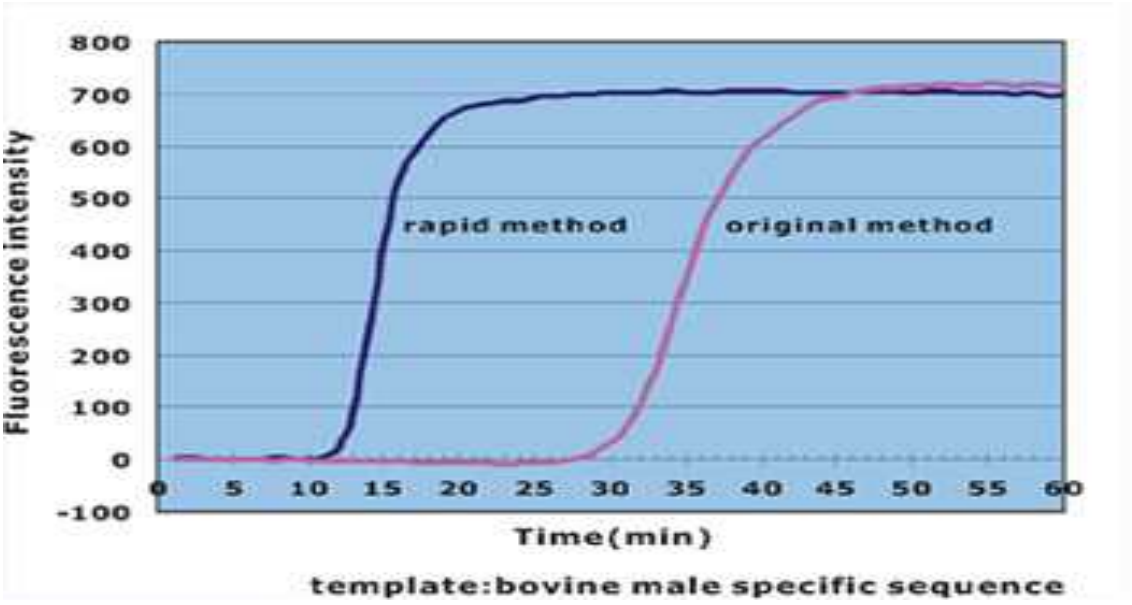
Principle of loop primers

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 (Eiken, 2005).

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.



3.12.9 Gyrase B gene (*gyrB*)

PCR, which permits the amplification of specific DNA sequences and multiplies even a single copy of a given DNA sequence by a factor of 10^{12}, 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 mycobacterial 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-kDa heat shock protein and the 38-kDa protein B antigen, the dnaJ gene, and insertion sequences such as IS6110 (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 (Holberg *et al*., 1999 and Patel *et al*., 2000). However, because of the extremely slow speed of the molecular evolution of 16S rRNA, the number of substituted bases between the 16S rRNA genes of closely related bacterial strains, such as those belonging to the *M. tuberculosis complex*, is either nonexistent or too small to differentiate between these species.

As an alternative to 16S rRNA analysis, Yamamoto and Harayama (1995) designed a set of PCR primers that allowed both the amplification of the *gyrB* gene, which encodes the subunit B protein of DNA gyrase (topoisomerase type II), and the rapid nucleotide sequencing of the amplified *gyrB* fragments from a wide variety of bacteria. They used these *gyrB* genes in the taxonomic classification of *Pseudomonas putida* and *Acinetobacter* strains. The rate of molecular evolution inferred from *gyr*B gene sequences is faster than that inferred from 16S rRNA gene sequences. For detection of *Mycobacterium spp*, Kasai *et al*., (2000) 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.

Analysis of RFLP allowed distinction of *M. tuberculosis, M. bovis, M. africanum, and M. microti*, Niemann *et al*., (2000) extended these observations and proposed a diagnostic algorithm of *gyrB*-RFLP patterns to differentiate *M. tuberculosis, M.*

africanum II from *M. africanum I, M. bovis* subsp bovis, *M. bovis* subsp caprae and *M. microti*.

3.12.10 LAMP for diagnosis of tuberculosis 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 non-mycobacterial 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^{o}C. 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.

To evaluate the usefulness of LAMP in Nepal, Pandey *et al*., (2008) compared the LAMP method with culture as gold standard for the detection of *M. tuberculosis*. They examined 200 sputum samples from patients suspected of having pulmonary tuberculosis. The sensitivity of LAMP in culture positive samples was 100% (96/96), and the specificity in culture negative samples was 94.2% (98/104). These results indicated that this LAMP method might prove to be a powerful tool for the early diagnosis of TB.

Song *et al*., (2005) reported LAMP method for detecting *Shigella* and Enteroinvasive *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 trypanosomiasis.

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.

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.

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 were 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 sequence, 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 < 10 bp. 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 *paracoccidioidomycosis,* 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%.

3.13 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^{o}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 by spectrophotometric analysis in 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 and 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 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.

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 this study is given in appendix I, II and III.

4.2 Methodology

4.2.1 Study site

This study was carried out from October 2006 to November 2007 at National TB Reference Lab German-Nepal Tuberculosis Project (GENETUP), Kalimati; Mycobacterial Research Laboratory, Anandaban Hospital; Everest International Clinic and Research Center (EICRC), Katmandu in collaboration with Osaka Perfectural Institute of public health, Japan.

4.2.2 Study population

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

A› Sputum smear positive TB patients (n=53): these were new cases of pulmonary tuberculosis visiting GENETUP who were sputum smear positive in microscopy.

B› Sputum smear negative patients (n=53): This group included the patients visiting GENETUP for checkup who had complains of chest problem and were negative in smear microscopy.

4.2.3 Sample collection

Sputum is the sample of choice in the study. The patients were given clear instruction about how to collect the sputum. During the sample and data collection, all the research objectives and the expected outcomes had been briefed and then verbal consent had been taken from each study participants. Among triplicate sputum samples (first, on the spot; second, early morning sample; and third, on the spot) collected at GENETUP, only early morning sample per patient was included in this study. All the samples were collected in leak proof, wide mouth, transparent, sterile and stopper plastic container. Adequate safety precautions were taken during the specimen collection to prevent the spread of infectious organism. Individuals known to be HIV and HBsAg positive, MDR, XDR, relapse, defaulter tuberculosis patients were excluded from this study.

4.2.4 Sample evaluation

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

4.2.5 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.

4.2.5.1 Sputum smears microscopy

An appropriate amount of the sputum sample was taken from the container with the sterile cotton swab and transformed to the clean, grease free slide. The specimen was spread on the slide to the size 2×1 cm and made it thin enough to be able to read through it. Then smear was allowed to 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.

Among the several methods of determining acid-fast nature of mycobacteria, fluorochrome method was included in this study. The numbered smear was placed on a staining rack and flooded with aura mine-O. It was then allowed to stain for 15 minutes. Then the smear was rinsed with distilled water and covered with 20% sulphuric acid for 5 minutes. The smear was rinsed again with distilled water and counterstained with 1% methylene blue for 2 minutes (Appendix III). After washing with distilled water, the smear was allowed to air-dry and examined directly under the UV microscope. Grading of microscopy is given in appendix IX (WHO, 1998a).

4.2.5.2 Sample concentration and culture

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

For culture, 0.1ml of concentrated sputum was inoculated into each of two culture tubes containing 2% Ogawa medium (Appendix II). The tubes were examined on 7^{th} day for rapid growers and weekly thereafter for slow growers. If any colonies were seen at stage, acid-fastness of bacilli was determined by smear examination of the growth. Negative report was given when no colonies appeared after observing weekly for 8 weeks. Grading of primary culture is given in appendix IX (WHO, 1998b).

4.2.5.3 Sample treatment for LAMP and PCR

An equal volume of 2% NaOH and 0.5% N-acetyl-L-cysteine (NaLC) (Appendix III) was added to the concentrated sputum sample. These were mixed by inverting for several times and left for 10 minutes to thinning out the sputum. The sample was then centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded again and the pellet and centrifuged again at 12,000 rpm for 15 minutes. The supernatant was discarded again and the pellet was resuspended in 50 mM Tris HCl of pH 8.3 equivalent to not less than $1/20^{th}$ of the original thinned sputum. Then it was heat inactivated at 80°C for 20 minutes in a dry heat block. After cooling to room temperature, an equal volume of chloroform was added and mixed by vortex and then stored in refrigerator (Sapkota *et al*., 2003).

DNA extraction

The DNA used for LAMP and PCR was extracted by freeze and boil method (Sapkota *et al.*, 2003; Woods and Cole, 1989).

Freeze and boil method

50 µl of sample was taken into an eppendorf tube. Then it was immersed into liquid nitrogen (-196°C) for one minute and it was heated to 100°C in the dry heat block for one minute. The alternate cooling and heating was repeated for 5 times to effect DNA extraction and the extracted DNA was stored in the refrigerator for LAMP and PCR.

DNA quantification

The estimation of the extracted DNA from the samples was carried out using spectrophotometric method. In this method, the sample was taken in eppendorf tube and diluted 100 fold by distilled water. Absorbance (OD) was measured in spectrophotometer at 260 nm and 280 nm by taking the sample in capillary cuvette, against distilled water as blank. OD 260 / OD 280 provide an estimate of purity of the nucleic acid, which is depicted as follows (Sambrook *et al*., 1989);

OD_1 = Absorbance at 260 nm

OD_2 = Absorbance at 280 nm

a = $OD_1 \times 50 * \times 100$

b = $OD_2 \times 50 * \times 100$

Where,

50 = constant factor for dsDNA (*10D = 50 µg DNA)

100 = Dilution factor

The pure preparation of DNA had a/b value of greater than 1.8. As the DNA concentration for PCR is recommended to 10ng/µl (Sambrook *et al*., 1989), the fold of dilution of sample required was calculated on this basis.

4.2.5.4 PCR

The multiple room approach (Appendix VI) for amplification containment was followed while performing PCR in this study.

Two primers synthesized by (Invitrogen T_m life technologies), a forward primer TB1-F (5'GAA CAA TCC GGA GTT GAC AA3') and a reverse primer TB2-R (5'AGC ACG CTG TCA ATC ATG TA3') were used to amplify a 372 bp region of the MPB70 gene of *M. tuberculosis*.

The sequential steps in PCR were as follows:

I. PCR reaction

After the treatment of sample as described before, PCR was performed in a total 50µl reaction mixture containing 20mM Tris-HCl (pH 8.0), 100mM KCl 2.5mM $Mgcl_2$, 2.5mM each of deoxynucleotides (dATP, dCTP, dGTP, dTTP), 30mM primer TB1-F, 30mM primer TB2-R, 5 units/µl *Taq* DNA polymerase, while final volume of PCR-mix was adjusted by adding sterilized distilled water in above solution.

The prepared PCR mix was vortex-mixed and dispensed in 44µl quantity on each labeled PCR tube (500µl) (Appendix V).

II. Sample loading

The sample loading was carried out in the gray room. In the PCR mix, contained in PCR tube, 6µl of sample was added while equal amount of positive sample and distilled water were added as positive and negative controls, respectively. It was vortex -mixed briefly and taken to PCR room.

III. Amplification

The loading PCR tubes were placed on the tube holder of the thermocycler. The cycle parameters (denaturation at 94^{o}C for 3 minutes; 35 amplification cycles- denaturation at 94^{o}C for 30 seconds, annealing of primers at 62^{o}C for 1 minute and primer extension at 72^{o}C for 1 minute: followed by the extension reaction at 72^{o}C for another 7 minutes) were used to run PCR samples (Jerzy *et al*., 1995).

IV. Gel preparation

Agarose gel (1.5%) was prepared in 1x Triacetate- EDTA buffer (Appendix III). The solubilization of agarose was effected by heating in boiling water for 40 minutes.The agarose, cooled to take warm, was poured on the moulder fitted with comb. Then, it was left for setting and immersed into the running buffer taken in electrophoresis unit.

V. Electrophoresis

Electrophoresis of DNA was carried out for the detection and visualization of amplified DNA.8µl of amplified product after fledging was mixed with 2 µl of Bromophenol blue (Appendix III). Then the 10 µl of mixture was loaded on each well of the gel. Electrophoresis was run at a voltage of 100V to achieve required distance (for about 60 minutes). The gel was taken out on a tray and brought to the staining table.

VI. Staining

200ml of distilled water was poured on the tray containing gel. The watering solution of ethidium bromide (200µl) (Appendix III) was added into the tray to give the final concentration of 0.56 μg/ml. The tray was shaken well and left for 1 hour .Then it was

washed twice with 200 ml distilled water and carried to the PCR room for UV illumination and photography.

4.2.5.5 Statistical analysis

For the comparison of PCR with standard culture as well as LAMP, the statistical analysis of tests were carried out by calculating sensitivity, specificity, positive and negative predictive values, percentage of false negative and percentage of false positive (Appendix XI).

4.2.5.6 LAMP

For rapid diagnosis of tuberculosis, mycobacterial DNA extracted from the concentrated sputum sample was used for LAMP with six primers specially designed targeting *gyr B* gene.

a. LAMP reaction

Number of cycle

LAMP was performed in a total 25 « l reaction mixture and resulting mixture was then incubated for one hour in thermocycler. Denaturation, primer annealing and extension steps were not required for this technique.

Temperature

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

Primers and enzymes

All together six primers were used during the study. These were: outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and loop primers (loop F and loop B). They recognize eight distinct regions on the target DNA. Sequence of primers used to diagnose *Mycobacterium tuberculosis complex* are given in appendix III.

LAMP was performed in a total 25 « l reaction mixture containing 10 x LAMP buffer, 14mM dNTPs, 5M 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.

b. Observation of results

LAMP amplicons in the reaction tube were directly detected with the naked eye by observing the change in color of the solution containing reaction mixture with SYBR Green I, a fluorescent dye which stains DNA and in large amount of amplicons give distinguished color.

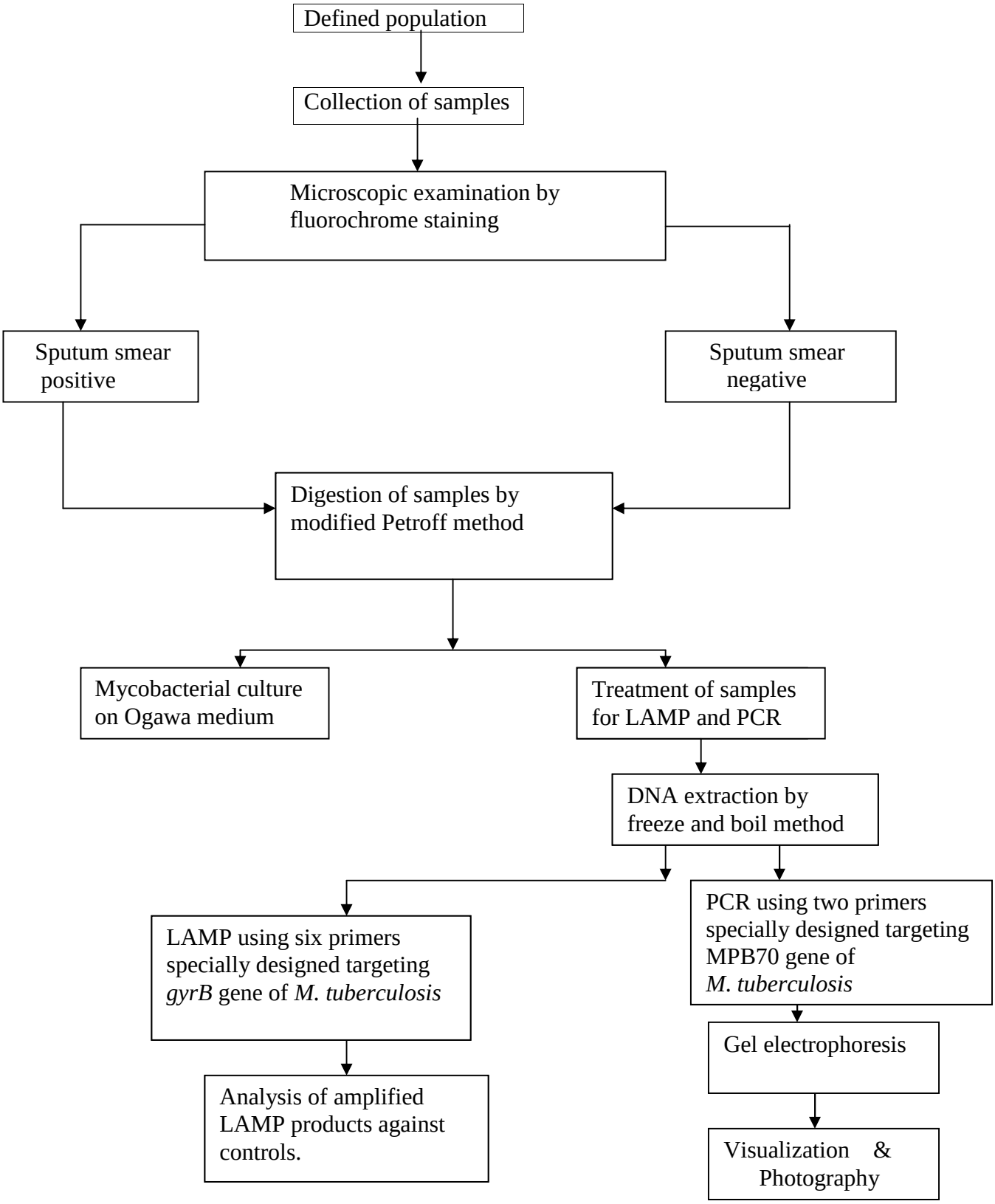
c. Interpretation of the results

The solution turned green in the presence of amplification product indicating positive result, while it remained orange with no amplification indicating negative result. Large amount of amplicons when bind with SYBR Green I, it fluoresces green indicating target gene amplification. Details of Standard Operating Procedure (SOP) in appendix V.

4.2.5.7 Statistical analysis

For the comparison of LAMP with standard culture as well as PCR, the statistical analysis of test were carried out by calculating sensitivity, specificity, positive and negative predictive values, percentage of false negative and percentage of false positive (Appendix XI).

Figure 2: Flow chart of methodology



CHAPTER V

5. RESULTS

In order to evaluate the different diagnostic techniques for rapid diagnosis of tuberculosis, a comparative study of LAMP, PCR and microscopy to detect *M. tuberculosis* in sputum sample, against culture as standard method was performed. The sputum samples from the patients belonging to two different study groups were collected in this study. Out of 106 samples, 53 were from smear positive TB patients and 53 were from smear negative patients respectively.

5.1 Distribution of total study cases (N=106) by age and sex

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

Age	Male		Female		Total	
Group (years)	No	%	No	%	No	%
<20	8	7.55	8	7.55	16	15.10
21-40	39	36.79	9	8.49	48	45.28
41-60	21	19.81	10	9.43	31	29.24
>60	9	8.49	2	1.89	11	10.38
Total	77	72.64	29	27.36	106	100

Among the studied 106 cases, 72.64% (n=77/106) were male and 27.36% (n=29/106) were female in the age group 14 to 81 years. This study showed that the highest number was seen in the age group 21-40 (45.28%), followed by 41-60 (29.24%) and so on.

5.2 Age and sex distribution of culture, LAMP and PCR positive cases

Out of 106 sputum samples, 53.77% (n=57/106) were culture positive, 52.83% (n=56/106) were LAMP positive and 55.66% (n=59/106) were PCR positive. Among total culture positive isolates, 73.68% (n=42/57) were male and 26.32% (n=15/57) were female. The highest number of culture positive cases were belonged to the age group 21-40. Out of 56 LAMP positive isolates, 71.43% (n=40/56) were male and 28.57% (n=16/56) were female. Similarly, out of 59 PCR positive isolates 71.19% (n=42/59) were male and 28.81% (n=17/59) were female.

5.3 Socio-demographic and disease characteristic of interviewed persons

A total 106 individuals were interviewed personally through structured questionnaire (Appendix XV). Among them 72.64% (n=77/106) were male and 27.36% (n=29/106) were female.

Among 106 interviewed individuals, 47.17% (n=50/106 were smoker; 52.83% (n=56/106) were non-smoker; 42.45% (n=45/106) had habit of taking alcohol and 57.55% (n=61/106) were non alcoholic; 27.36% (n=29/106) had prior presence of TB in their family members, whereas 72.64% (n=77/106) had not the prior presence of TB in their family members.

Out of total interviewed patients, 70.75% (n=75/106) were immunized by BCG vaccination, where as 29.25% (n=31/106) were non-vaccinated. While asking the patients about the symptoms of TB, 74.52% (n=79/106) had the symptoms of fever, 68.86% (n=73/106) had chest pain, 86.79% (n=92/106) had cough, 71.69% (n=76/106) had weight loss, and 34.90% (n=37/106) had haemoptysis respectively.

Table 2: Socio-demographic and disease characteristic of interviewed persons (n=106)

Socio-demographic characteristics	No. of patients	Percentage
Smoking		
Smoker	50	47.17%
Non-Smoker	56	52.83%
Alcohol		
Habit of drinking alcohol	45	42.45%
Not	61	57.55%
Family history of TB		
Present	29	27.36%
Absent	77	72.64%
BCG		
Vaccinated	75	70.75%
Non-Vaccinated	31	29.25%
Symptoms		
Fever	79	74.52%
Chest pain	73	68.86%
Cough	92	86.79%
Haemoptysis	37	34.90%
X-Ray		
Positive	55	51.89%
Negative	51	48.11%

5.4 Laboratory result of samples

In this study, a total of 106 sputum samples were collected from 106 patients belonging to two different categories and examined by microscopy, culture, LAMP and PCR.

5.4.1 Study group A

This group includes 53 smear positive PTB patients, 94.33% (50/53) were positive by radiological examination (chest X-ray) and the rest 5.67% (3/53) were negative.

Out of 53 (100%) fluorochrome staining positive sputum samples collected from these PTB patients, 100% (53/53) samples were positive by culture on Ogawa medium. Upon testing by LAMP using specific six primers, 96.22% (51/53) samples were found to be positive while the remaining 3.77% (2/53) samples (2906B, 824B) were negative. Similarly, upon testing by PCR using specific two primers, 96.22% (51/53) samples were found to be positive while the remaining 3.77% (2/53) samples (2906B, 824B) were negative.

Table 3: Comparative results of fifty-three smear positive sample with culture, LAMP and PCR.

Stain	Culture	LAMP	PCR	Number (%)
Positive	Positive	Positive	Positive	51 (96.22%)
Positive	Positive	Positive	Negative	0
Positive	Positive	Negative	Positive	0
Positive	Positive	Negative	Negative	2 (3.77%)

Among the total of 53 microscopy positive samples majority of samples i.e. 96.22% (51/53) were positive by culture, LAMP and PCR. Similarly, 3.77% (2/53) LAMP and PCR negative samples (2906B and 824B) were found to be culture positive.

5.4.2 Study group B

In this group, out of 53 smear negative patients with complain of chest problem, 9.43% (5/53) were found to be positive by X-ray and the remaining 90.57% (48/53) were negative.

Among 53 (100%) fluorochrome staining negative sputum samples collected from these cases, 7.54% (4/53) (258B, 313B, 444B, 5570B) were positive by culture where as the remaining 92.45% (49/53) were negative. Here the result of LAMP for these microscopy negative samples showed 9.43% (5/53) samples (258B, 313B, 323B, 444B, 5570B) were positive and 90.56% (48/53) were negative. Similarly, 15.09% (8/53) smear negative samples (258B, 313B, 323B, 335B, 393B, 453B, 444B, 5570B) showed PCR positive while 84.90% (45/53) were negative.

Table 4: Comparative results of fifty-three smear negative samples with culture, LAMP and PCR

Stain	Culture	LAMP	PCR	Number (%)
Negative	Positive	Positive	Positive	4 (7.54%)
Negative	Positive	Positive	Negative	0
Negative	Positive	Negative	Negative	0
Negative	Negative	Positive	Positive	1 (1.88%)
Negative	Negative	Negative	Positive	3 (5.66%)
Negative	Negative	Negative	Negative	45 (84.90%)

Similarly, out of 53 smear negative samples, 7.54% (4/53) were positive by culture. Remarkable points are that there were 1.88% (1/53) sample which was positive by LAMP and PCR but stain and culture negative, similarly 5.66% (3/53) were positive by PCR but negative by stain, culture and LAMP. Majority of samples 84.90% (45/53) were negative by all tests.

To sum up, a total of 106 samples were collected and examined from the total of 106 patients to two different categories. Among all, 53 (50%) were microscopy positive and 53 (50%) were microscopy negative samples; 52.83% (56/106) were LAMP positive and 47.16% (50/106) were LAMP negative; while 55.66% (59/106) were PCR positive and 44.33% (47/106) were PCR negative. Similarly 53.77% (57/106) were culture positive and 46.22% (49/106) were culture negative (Appendix XIV).

Table 5: Results of one hundred six samples by microscopy, culture, LAMP and PCR

Microscopy		Culture		PCR		LAMP	
+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
53 (50%)	53 (50%)	57 (53.77%)	49 (46.22%)	59 (55.67%)	47 (44.33%)	56 (52.83%)	50 (47.17%)

5.5 Evaluation of the Tests

In this study, the clinical performance of PCR, LAMP and fluorochrome staining for the rapid detection of *M. tuberculosis* in sputum samples was determined by comparing these techniques with those of standard culture.

Among 106 samples studied by microscopy and culture, 53 were positive by both, whole 4 were positive by culture but negative by microscopy. Both test revealed 49 samples to be negative, but 0 cases were negative by culture and positive by microscopy. With reference to culture, the microscopy had sensitivity 92.98%, specificity 100%, and predictive value of positive test 100%, predictive value of negative test 92.5%, percentage of false negative 7.01% and percentage of false positive 0% respectively (Appendix XI)

Table 6: Comparison of microscopy with reference to culture

	Test and results	Culture		Sensitivity	Specificity	PV+	PV-	False -ve	False +ve
Microscopy		+ve	-ve						
	Positive	53	0	92.98%	100%	100%	92.45%	7.01%	0%
	Negative	4	49						

Out of 106 samples subjected to culture and LAMP for the diagnosis of TB, 55 samples were positive by both tests and 2 were positive only in culture, while 48 were negative in both tests and 1 was negative only in culture. While comparing the LAMP with culture as a gold standard, the sensitivity of LAMP was 96.49%, specificity was 97.95%, predictive value of positive test was 98.21%, predictive value of negative test was 96%, percentage of false negative was 3.50%, and percentage of false positive 2.04% respectively (Appendix XI)

Table 7: Comparison of LAMP with reference to culture

	Test and results	Culture		Sensitivity	Specificity	PV+	PV-	False -ve	False +ve
LAMP		+ve	-ve						
	Positive	55	1	96.49%	97.95%	98.21%	96%	3.50%	2.04%
	Negative	2	48						

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

Culture and PCR employed for diagnosing tuberculosis in 106 samples showed only 55 of them to be positive by both tests and 2 to be positive by culture but negative by PCR; 45 were negative by both tests and 4 samples were found to be culture negative but PCR positive. These findings gave the sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive as 96.49%, 91.83%, 93.22%, 95.74%, 3.50% and 8.16% respectively.

Table 8: Comparison of TB-PCR with reference to culture

	Test and results	Culture		Sensitivity	Specificity	PV+	PV-	False -ve	False +ve
PCR		+ve	-ve						
	Positive	55	4	96.49%	91.83%	93.22%	95.74%	3.50%	8.16%
	Negative	2	45						

Out of 106 samples subjected to LAMP and PCR for diagnosis of TB, 56 were found positive in both tests and 3 were positive only in PCR, while 47 were negative in both tests and none were found to be PCR negative and LAMP positive. From these results, LAMP for TB was compared with PCR by calculating sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive. The values were found to be 94.9%, 100%, 100%, 94%, 5.08% and 0% respectively.

Table 9: Comparison of TB-LAMP with reference to TB-PCR

	Test and results	PCR		Sensitivity	Specificity	PV+	PV-	False -ve	False +ve
LAMP		+ve	-ve						
	Positive	56	0	94.91%	100%	100%	94%	5.08%	0%
	Negative	3	47						

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Tuberculosis is an important public health problem worldwide and its control should not only mean to the clinically proven cases of TB but more important to identify early those individuals who are in the early stages or at risk of developing the disease. An important key to successful control of TB is thus timely diagnosis (Sritharan and Sritharan, 2000). Rapid and accurate diagnosis of tuberculosis is the cornerstone of global tuberculosis control programmes. With increasing incidence of tuberculosis epidemics, the low sensitivity and the length of time taken by traditional diagnostic modalities have hampered the efforts to interrupt disease transmission (Singh and Parija, 1998).Conventional methods such as acid fast smear, culture and radiological examination have been still remain the methods of diagnosis of human TB in many developing countries even today. On the other hand, PCR and other nucleic acid methods are widely used for the detection of *Mycobacterium tuberculosis* in clinical specimens because of the test's rapidity, sensitivity and specificity (Noordhoek *et al*., 1994). In the present study, LAMP and PCR techniques were performed on sputum samples for the amplification of *gyrB* gene and MPB70 gene of *M. tuberculosis* respectively. Additionally, the sensitivity specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive of PCR, LAMP and microscopy were evaluated in reference to cultural technique as "Gold Standard".

Although relatively rapid, simple and inexpensive, sputum microscopy suffers from a major drawback of low sensitivity i.e. the detection limit for microscopy is about 5,000-10,000 bacteria per ml and is nonspecific for species identification and differential diagnosis. Isolation of the organism by culture and subsequent identification by biochemical test is time-consuming. Due to these shortcomings, we need to have the

novel molecular techniques that combine the rapidity of microscopy and the sensitivity of culture such as LAMP, PCR and that can identify the mycobacterial species would be great help to the clinician during the initial treatment of the patient. Though these techniques are not used routinely in Nepal, some investigators reported its feasibility (Sohn *et al*., 2003; Poudel, 2005; Adhikari, 2007). In this present study LAMP and PCR techniques are performed to provide the new possibilities for diagnosis of tuberculosis in Nepal.

In this study, males (72.64%) were found to be more infected than females (27.36%) and the result was found to be significant statistically. This finding was in agreement with the findings of the Shrestha (2006); Poudel (2005); Adhikari (2007). In similar study conducted in Italy, Ponticeellio *et al.*,(2001) reported , 82.2% male and 17.8% of female among 90 active pulmonary TB cases; Similarly in the study of National Tuberculosis Control Programme, Nepal, it has been reported that 69.8% male and 30.2% female of TB cases among 14,384 newly diagnosed TB cases during 2002/2003. These all data are in favour and figure of Nepal is also favour of this study. This does not however reflect an increase in the occurrence of disease in males, since in present study the attendance of females is lower than males (Table 1). TB was not diagnosed in the PTB suspects below 14 years. On the other hand females make less report than male in health care facilities, because of social pressure or stigma and more exposure to external environment than females for their job and other activities.

In the present study maximum number of culture positive was observed in the economically most productive age group 21-40 which is in agreement with Poudel (2005); Shrestha (2006).

Among 53 PTB patients with a positive acid-fast stain on sputum smear, 94.33% (50/53) were positive by chest X-ray while the remaining were negative. The X-ray positive results are due to cavities usually develops because of immune response to the tubercle bacilli leading to destruction of lung tissue. The X-ray negative result may be due to the people who don’t have fully functioning immune system like in HIV cases,

where there is less tissue destruction and hence lung cavitations. Another reason might be due to the patients who were suffered from bronchial or tracheal tuberculosis in whom infectious agent might not be spread to the lungs.

Patients with negative acid fast staining on sputum samples were recruited into group B where 9.43% (5/53) were positive in chest X-ray and the rest were negative. Abnormalities on chest X-ray may be suggestive of, but are never diagnostic of TB because a number of other bacterial conditions (such as pneumonia or abscess) or non-bacterial processes (fungal diseases, carcinoma, sarcoidosis or pneumoconiosis) can produce similar images. The vast majority of patients (over 90%) with cavitary PTB are sputum smear positive (WHO, 1997).

Among 53 smear positive cases; all were positive while culturing on Ogawa medium. However, among 53 smear negative cases, 4 were positive by culture. In this study, none of the fluorochrome staining positive samples showed negative results in culture, thus showing 100% specificity. This means that as long as the stain gives positive result, it is not necessary to do further test for the purpose of diagnosis. However, due to the increase of drug resistant bacilli, culture is recommended to test for the sensitivity of antituberculosis drug (Sohn *et al*., 2003). Culture methods are highly sensitive than microscopy for the detection of bacilli as approximately 10-100 mycobacterium per ml of sample is required for positive result while 10^4 organisms per ml of sputum is required to be seen by microscopic examination. Garay (2000) reported that the proportion of positive sputum smear cases in PTB-AIDS complex is even lower.

While in smear negative cases, the sensitivity of microscopy in reference to culture was found to 92.98%. This indicates the sensitivity of microscopy was not good as its specificity. However, considering the availability and technical easiness, microscopy method could be still used as the method of choice in the first line diagnosis of tuberculosis (Sohn *et al*., 2003). The finding of the present study was in agreement with the findings of Poudel (2005) and Adhikari (2007) in smear negative cases. However, this was not in agreement with smear positive cases as 6.93% (7/101) smear positive

cases showed no growth on culture on Ogawa medium by Poudel and 5.55% (3/54) smear positive cases showed no growth on culture on L-J medium by Adhikari respectively. Nagesh *et al*., (2001) reported that microscopy positive specimens fail to yield mycobacterium on culture perhaps due to harsh chemical treatment used in decontamination or the presence of nonviable mycobacterium in partially treated patients.

Among 53 fluorochrome staining positive and culture positive cases, 51 were positive by PCR and remaining two samples were negative by PCR. Similarly, among 53 fluorochrome staining negative cases, 4 were positive by culture and 8 were positive by PCR. In this present study, two stain and culture positive samples didn't give positive results in PCR. This was in agreement with the findings of Sohn *et al*., (2003) and Iwamoto *et al*., (2003). In this study, 4 stain and culture negative specimens gave positive results in PCR. The negative result in PCR might be due to MOTT which was not targeted by this technique. It was also conceivable that those *M. tuberculosis complex* strain might be particularly sensitive to genome disruption during the extraction phase. Those result presumed to result from either a laboratory error or uneven aliquot distribution as true positive. It was also possible that there had been a base pair deletion or mutation that had interfered with primer attachment (Jorgen *et al*., 1996).The investigators suggested the use of back-up PCR with a separate primer pair for such specimens. However, in the present study backup PCR was not performed for culture positive and PCR negative samples.

Given the high prevalence of TB in Nepal, it is possible that these four false positive (microscopy and culture negative but PCR positive) represent nonviable organisms because of overzealous decontamination, latent infection, and cross-contamination of preliquified sputum from another positive sample. Another explanation is that these results may represent DNA contamination, a constant problem with PCR method. However, the negative controls were employed to exclude the problem were all negatives, which ruled out the possibility of contamination.

The sensitivity and specificity of PCR in comparison to culture as gold standard were found to be 96.49% and 91.83% respectively. The sensitivity was in agreement with the sensitivity reported by Suh *et al*., (1994).

Jorgen *et al*., (1996) reported the overall sensitivity of PCR of 82.0% in specimen evaluations and 85.7% in patient evaluations. Additionally, the overall specificity in specimen evaluations was 96.1%.

Douglas and Janis (1995) reported that clinical sensitivity of PCR compared with that of culture has been reported to be from 74% to greater than 100% with actual detection limits of <1 to 100 cfu. In some instances, test production and diagnostic testing in the same facility can lead to poor sensitivity because of the lack of assay optimization or low specificity because of amplicon contamination.

Wilson *et al*., (1993) suggested that the sensitivity and specificity of PCR also depends upon the method of extraction of DNA from the sputum sample. They reported that the PCR of samples prepared by the chatrope-silica method had a sensitivity of 75% and specificity of 100% whereas PCR of samples prepared by the chloroform method had a sensitivity of 92% and a specificity of 99% when compared with the sensitivities and specificities of the combined classical microbiological methods for the diagnosis of TB.

Among 53 PTB patients with a positive acid fast stain on sputum smear and culture positive too, LAMP was positive for 51 samples while it was negative for the remaining 2 samples. While out of 53 smear negative samples from the patients of group B, LAMP was positive for 5 samples and it was negative for the rest of the samples. The finding reveals sensitivity of LAMP over microscopy. When the result of culture on Ogawa medium obtained, only four samples were found to be positive. These four culture positive samples were also positive by LAMP confirming the LAMP result with culture. These findings emphasized the sensitivity of the LAMP over culture. It is remarkable that LAMP positive sample was found to be negative by culture. 48 microscopy negative samples were found to be negative by both LAMP and culture. This finding highlighted the specificity of LAMP because all the samples that were

negative for tubercle bacilli microscopically and culturally were also negative by LAMP. Thus, LAMP combines the rapidity of microscopy with specificity of culture.

While comparing the LAMP with culture as gold standard, the sensitivity specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive were 96.49%, 97.95%, 98.21%, 96%, 3.50% and 2.04% respectively. This demonstrates a high sensitivity, specificity and predictive value of positive test and predictive value of negative test of LAMP as compared with that of culture. Percentage of false negative and percentage of false positive are also very low indicating the higher accuracy of the test. The finding of the present study was in agreement with the previous findings of Poudel (2005) who reported the sensitivity and specificity of LAMP as 97.0% and 94.12% respectively in comparison to culture as gold standard. However, the specificity of LAMP was reported lower than these findings by Adhikari in 2007, while the sensitivity was similar. Cathrina *et al*., (2007) reported that the sensitivity of LAMP in smear and culture positive sputum specimen was 97.7% and sensitivity in smear negative and culture positive specimen was 48.8%. The specificity in culture negative sample was 99.0%.

Among smear positive cases, the findings of LAMP and PCR were identical i.e. both the techniques gave positive results among 51 cases out of 53. However, among 53 smear negative cases, LAMP gave positive result among 5 whereas PCR gave 8 positive results. Three samples that were negative by LAMP were positive by PCR. In this study the PCR protocol for DNA extraction from sputum specimens was used. When a more compatible DNA extraction method for the LAMP assay is developed, it will increase the rate of detection of *Mycobacteria* in clinical specimens. The sensitivity of LAMP assay on sputum samples is slightly lower than that of PCR. The findings of present study was in agreement with the findings of Iwamoto *et al*., (2003) who reported that when purified DNA was used for analysis by LAMP and Amplicor, both the techniques had an equivalent detection limit. But, when sputum specimens were analyzed, the sensitivity of LAMP assay was slightly lower than that of Amplicor. They also reported that the LAMP reaction with a 60 min-incubation and visual inspection has a sensitivity

equivalent to that of Amplicor test. Both methods showed a detection limit of 5 to 50 genomes per test for the three mycobacterial species i.e., *M tuberculosis complex, M. avium* and *M. intracellulare*. The sensitivity of the LAMP assay was 10 to 100 times lower when the reaction time was shortened to 35 minutes.

The sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive of LAMP were found to be 94.91%, 100%, 100%, 94%, 5.08% and 0% respectively. The present study revealed that LAMP is highly specific for the target sequence. This is attributable to recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences during the later stages of the LAMP reaction. This partly alleviates the general problem of backgrounds associated with all nucleic acid amplification methods (Notomi *et al.*, 2000).

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

Saito *et al*., (2005) reported 100% concordance between LAMP and real-time PCR for the rapid detection of *Mycoplasma pneumoniae* from nasopharyngeal swabs. The investigator also reported LAMP assay as easier, rapid and inexpensive and recommended for its application for the diagnosis of *M. pneumoniae* infection in clinical laboratory.

Hara-Kudo *et al.*, (2005) used LAMP assay to detect *Salmonella* within 60min. 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 the sensitivity of the LAMP-based method for the detection of 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 HSV 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%.

6.2 Conclusion

In conclusion, this study showed that PCR and LAMP tests are not necessary for the diagnosis of smear positive cases. However PCR and LAMP could be possible tool for confirmatory diagnosis of the smear negative cases, which show clinical symptoms of TB. Among the different diagnostic tools, LAMP assay is more advantageous than other techniques performed in this study 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 the remaining issues such as sample preparation, nucleic acid extraction, and cross-contamination controls are addressed.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

PTB is major public health problem in developing countries. The highest priority for TB control is the identification and cure of infectious cases. Sputum Smear microscopy is still the cornerstone of TB diagnosis in the developing world. The present study was conducted in GENETUP hospital, Mycobacterial Research laboratory, Anandaban Hospital, Everest International Clinic and Research Centre in collaboration with Osaka Perfectural Institute of public health, Japan with the aim to evaluate four different diagnostic techniques (Microscopy, Culture, PCR and LAMP) for the diagnosis of PTB. In this study culture was employed as a gold standard.

In this study, LAMP and PCR with targeting to *gyrB* gene and MPB70 gene of *Mycobacterium tuberculosis* were applied for direct detection of *M. tuberculosis* in sputum samples. A total of 106 patients belonging to two different categories were included in this study. Among them 55 were positive in X-ray and the rest were negative. Out of 106 sputum samples collected from these patients, 53 samples were microscopy positive, 57 were culture positive, 56 were LAMP positive, and 59 were PCR positive.

Of 53 (100%) fluorochrome staining positive sputum samples collected from PTB patients, all the samples of 53 (100%) were positive by culture on Ogawa medium. upon testing by LAMP using specific six primers, 96.22% (51/53) were found to be positive while the remaining 3.77% (2/53) were negative. Identical results were obtained upon testing with PCR by using specific two primers, 96.22% (51/53) were found to be positive and 3.77% (2/53) were negative.

Among the total of 53 microscopy positive samples, the majority of samples i.e. 96.22% (51/53) were positive by culture, LAMP and PCR. Similarly, 3.77% (2/53) LAMP and PCR negative samples were found to be culture positive.

Of 53 (100%) fluorochrome staining negative sputum samples collected from 53 smear negative patients with complain of chest problem, 7.54% (4/53) samples were positive by culture where as the remaining 92.45%(49/53) were negative. Here, the result of 0LAMP for these microscopy negative samples showed 9.43% (5/53) were positive and 90.56% (48/53) were negative. Similarly, 15.09% (8/53) smear negative samples showed PCR positive while 84.90% (45/53) were negative.

Similarly, out of 53 smear negative samples, 7.54% (4/53) samples were positive by culture LAMP and PCR.While the majority of samples 84.90% (45/53) were negative by all tests. Remarkable points are that there were 1.88% (1/53) samples which were positive by LAMP and PCR but stain and culture negative, similarly 5.66% (3/53) were positive only by PCR but negative by stain, culture and LAMP.

While comparing the microscopy with culture as gold standard, the sensitivity of microscopy was 92.98%, specificity 100%, predictive value of positive test 100%, predictive value of negative test 92.45%, percentage of false negative 7.01% and percentage of false positive 0% respectively. Similarly comparing the PCR with culture as gold standard, the sensitivity 96.49%, specificity 91.83%, and predictive value of positive test 93.22%, predictive value of negative test 95.74%, percentage of false negative 3.50% and percentage of false positive 8.16%.

While comparing the LAMP with culture as gold standard, the sensitivity of LAMP was 96.49%, specificity 97.95%, predictive value of positive test 98.21%, predictive value of negative test 96%, percentage of false negative 3.50% and percentage of false positive 2.04%, however; with reference to PCR, the LAMP had sensitivity 94.91%, specificity 100%, predictive value of positive test 100%, predictive value of negative test 94% of false negative 5.08% and percentage of false positive 0% respectively.

7.2 Recommendations

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

1. LAMP can be used for the diagnosis of *Mycobacterium tuberculosis* in sputum samples because of its rapidity, sensitivity and specificity even in clinical laboratories with no specific equipment.
2. Those samples for which there was disagreement between PCR and culture results are recommended to test for PCR inhibitors and should perform back up amplification with a separate primer pair.
3. In case of smear and culture positive but PCR and LAMP negative samples, PCR and LAMP should be performed for MOTT.
4. Because of its high sensitivity and specificity, LAMP can be used as the supplementary test for confirmatory diagnosis of clinically suspected and sputum smear negative cases

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APPENDICES

Appendix I: Materials

A) Bacteriological media

2% Ogawa 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
Sodium glutamate
Taq DNA polymerase
Tris HCl

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
Incubator	:	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	Staining rack
Bunsen burner	Spirit lamp
Forceps	Soaps
Gloves	Tube holder
Labeling stickers	Tissue paper

Appendix II: Bacteriological media

2% Modified Ogawa Medium

1. Preparation of salt solution — 500ml/flask

Potassium dihydrogen phosphate	2.0 gm
Magnesium citrate	0.1 gm
Sodium glutamate	0.5 gm
D/W	100ml

   - Mix well and heat at 100°C for 30 minutes in a water bath (or autoclave at 121°C for 15 minutes).
   - Add glycerol 4 ml into the salt solution (while it is hot).
   - Add 4 ml 2% malachite green solution.

2. Preparation of whole egg homogenate
   - Wipe off eggshell with spirit cotton.
   - Break down the egg into a plate to check the decomposition.
   - Transfer the egg into the beaker (500ml).
   - Homogenize the egg with a pair of chopsticks until the egg become watery.
   - Place the two layers of sterile gauze pieces on the funnel.
   - Filter the egg homogenate till to get 200ml.
3. Mix 1 with 2 (Raw Modified Ogawa Medium)
4. Distribution of raw medium
   - Dispense the medium 6 ml into each tube (avoid bubble formation).
5. Inspissations
   - Arrange the tubes in slant position and coagulate them at 90°C for 1 hr with caps closed loosely.
6. Store at 4°C -6°C with caps closed tightly.

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. NaOH solution

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

NaOH pellet	40 gm
D/W	100 ml

Working solution: 4%NaOH(500ml)

40%NaOH	50ml
D/W	450ml

B. N-acetyl L-cysteine (NaLC) solution

Stock solution: 10M

Working solution: 2% NaOH, 0.5% NaLC solution (1000µl)

40%NaOH 50µl

10M NaLC 50µl

D/W 900 µl

C.Tris HCl

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

Take 12.1gm of Tris and dissolve in 60 ml (pH as 10.70). Maintain pH at 8.3 by adding HCl. Make final volume up to 100ml.

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

1 M tris HCL of pH 8.3 50µl

D/W 950 µl

3.Solution for DNA purification

A. Phenol (Central Drug House(P) Ltd., India)

B. Chloroform (Merck Company)

4. Reagents and solution for LAMP and PCR

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

A. For LAMP

LAMP Buffer

$MgSO_4$

Betaine (N,N,N-trimethylglycine)

Deoxyribonucleoside triphosphate (dNTPs)

Bst DNA polymerase.

Template DNA

SYBR Green I

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/umol)	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/umol)	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 GCA TGT CTT TA

Molecular weight (µg/mol)	6075.0	Primer length	20
Millimolar extinction coeff. (OD/umol)	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/umol)	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/umol)	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/umol)	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

B. For PCR

1. 10 x Buffer

2. dNTPs (dATP, dCTP, dGTP, dUTP)

3. *Taq* DNA polymerase

4. Template DNA

5. Primers (Invitrogen T_m life technologies)

Primer 1:		Primer Number**:**	D6918CO4(CO4)
Primer name: TB1-F		Primer Length:	20
Researcher: Suzuki Y		Scale of Synthesis:	50n mol
Sequence (5' to 3') GAA CAA TCC GGA GTT GAC AA			
Molecular weight (µg/µmole):	6160.0	µg per OD:	25.7
Millimolar Extinction Coeff:(OD/µmol)	238.9	nmoles per OD	4.1
Purity	Desalted	OD's	10.40
Tm (1 M Na+)	66	µg's	268.16
Tm (50 mM Na+)	45	nmoles	43.6
% GC	45	Coupling Eff.	99%

Primer 2:		Primer Number**:**	D6918CO5(CO5)
Primer name: TB1-R		Primer Length:	20
Researcher: Suzuki Y		Scale of Synthesis:	50n mol
Sequence (5' to 3') AGC ACG CTG TCA ATC ATG TA			

Molecular weight (µg/µmole):	6102.0	µg per OD:	27.4
Millimolar Extinction Coeff:(OD/µmol)	222.5	nmoles per OD	4.4
Purity	Desalted	OD's	10.50
Tm (1 M Na+)	66	µg's	287.96
Tm (50 mM Na+)	45	nmoles	47.2
% GC	45	Coupling Eff.	99%

5. Reagents and solution for gel electrophoresis

A. Agarose

Supplied by Sigma Company.

B. 1x TAE
C. Tris EDTA (10mM Tris, 1mM EDTA**)**
D. Bromophenol Blue (BPB)(C_{19} H_9 Br_4 O_5 Na, FW 691.9) (Sigma Company)

Bromophenol blue (3',3'', 5', 5''-tetrabromophenolsulfonephthalein) sodium salt.

E. DNA molecular weight markers (Sigma Company)
F. Ethidium bromide (Sigma Company)

C_{21} H_{20} N_2 Br FW: 394.3

Stock solution: 10mg/ml, i.e., 50 mg in 5ml distilled water.

Working Solution: In 280 µl of 10 mg/ml, add 4720 µl of distilled water that gives 560µg/ml.

Appendix IV: Primers used by Iwamoto et al. (2003)

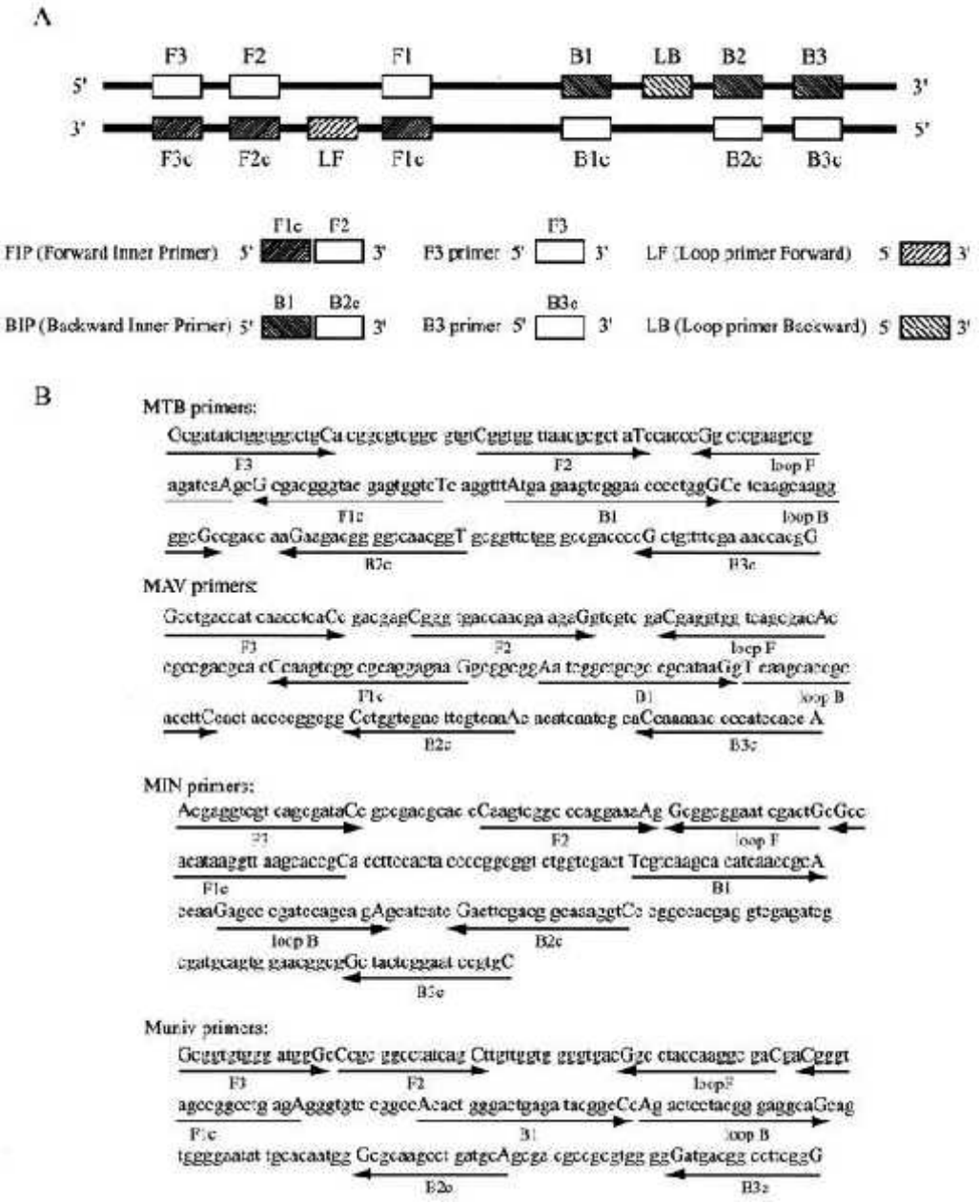


Figure: (A) Schematic representation of primers used by Iwamoto *et al.* (2003). Construction of the inner primers FIP and BIP is shown. F1c and B2c complementary sequences of F1 and B2, respectively. (B) Nucleotide sequences of *gyrB* and 16SrDNA used for designing the primers. Recognition sequences of the primers are shown between capital letters. A right arrow indicates that a sense sequence is used for the primers. A left arrow indicates that a complementary sequence is used for the primer (Iwamoto *et al.*, 2003).

Appendix V: Standard Operating Procedure for LAMP and PCR

General reaction mixture for LAMP (Total 25 µl)

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^{o} C for 60 min.

↓

Visual inspection of LAMP amplified products against controls.

General reaction mixture for PCR (Total 50 µl)

For 1 sample tube		
S.N.	Mixture components	Volume (50µl)
1.	dH_2O	21.5 µl
2.	10x PCR Buffer	5 µl
3.	2.5mM $MgCl_2$	5 µl
4.	dNTP mixture (2.5mM each)	8 µl
5.	30mM Primer TB1-F	2 µl
6.	30mM Primer TB2-R	2 µl
7.	TaKaRa LA Taq polymerase (5 units/µl)	0.5 µl
8.	DNA Sample	6 µl

↓

Thermal cycle

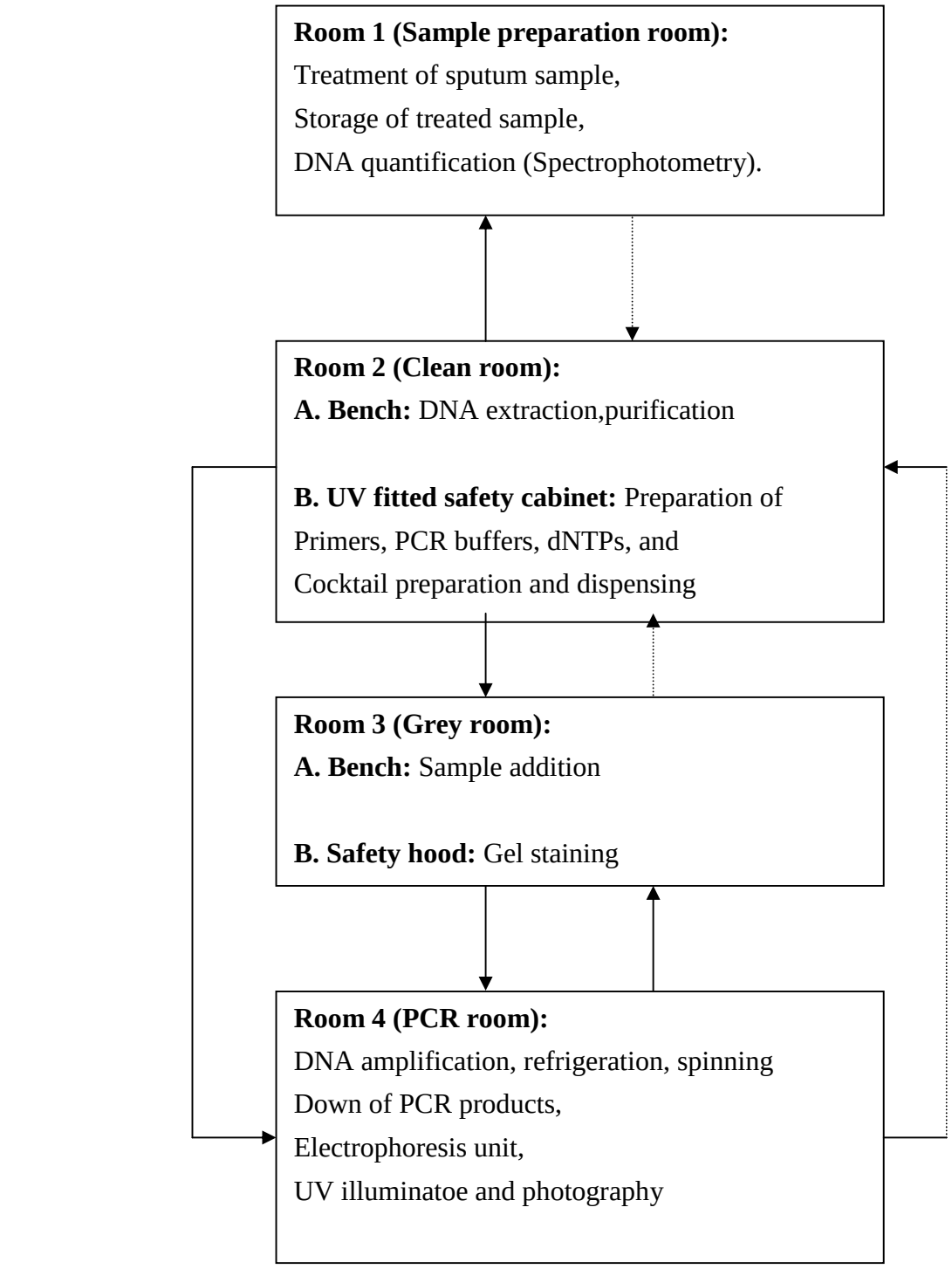
↓

Electrophoresis

↓

Visualisation and Photography

Appendix VI: The multiple room approach followed in PCR (Sapkota, 2003)



Appendix VII: Clinical utility of PCR in diagnosis of Tuberculosis

Smear	PCR	Clinical suspicion	Comment
Positive	Positive	High/Low	Diagnosis : active TB
Negative	Negative	High/Low	Cannot exclude active TB
Negative	Positive	High	Suspect *M. tuberculosis* start treatment and review therapy when culture results are final
Negative	Positive	Low	Active TB, old TB, or contaminant: consult specialist
Positive	Negative	High	*M. tuberculosis* or nontuberculous mycobacteria: start therapy and review when ulture results are final
Positive	Negative	Low	Most likely nontuberculous mycobacteria but cannot exclude *M. tuberculosis* : consider initiating TB therapy until culture results are final

Source: Martin and Lazarus (2000).

Appendix VIII: DNA molecular weight markers

SIGMA GENOSYS

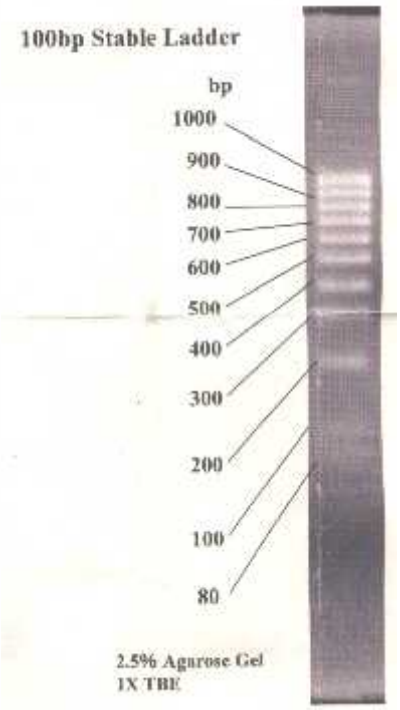
Stable 100bp DNA Ladder

MBMA100BP-S
50ng/ μl, 0.05mg
研究用試薬

SIGMA Genosys Japan K.K.
E-mail : genosys@genosys.jp
http://www.genosys.jp/

10μl泳動した場合の各バンドのDNA量は、

1000bp	72 ng
900bp	61 ng
800bp	61 ng
700bp	56 ng
600bp	53 ng
500bp	51 ng
400bp	48 ng
300bp	43 ng
200bp	36 ng
100bp	12 ng
80bp	8 ng
Total	500 ng



Appendix IX: Grading of Microscopy and culture result

The grading of microscopy was based on **WHO/ IUATLD** following criteria:

Number of AFB observed/ field	Report
100 and more AFB per field	4+
19-99 AFB per field	3+
1-9 AFB per field	2+
Less than 1 AFB per field but more than 10 per slide	1+
1-10 AFB per slide	Doubtful
No AFB seen	0

The inoculated slopes were examined for growth. The grading of primary culture was based on WHO/ IUATLD following criteria:

Confluent growth	4+
More than 200 colonies, but not confluent growth	3+
100-200 colonies	2+
50-100 colonies when less than 50 colonies; the number of colonies per slope i.e. the mean of the number on the two slopes.	1+
No growth	-

All suspected colonies were examined by fluorochrome method to confirm the colonies of mycobacteria.

Appendix X: Screening test result by diagnosis

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

The letter" a" denotes those individuals found positive on the test who have the condition or disorder being studied (i.e., true positives). The group labeled "b" includes those who have a positive test result 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 XI: Statistical analysis of test

A) Comparison of Microscopy with reference to culture

	Test and result	Culture		Total
		Positive	Negative	
Microscopy	Positive	53 (a)	0(b)	53 (a+b)
	Negative	4 (c)	49 (d)	53 (c+d)
	Total	57 (a+c)	49 (b+d)	106 (a+b+c+d)

Sensitivity= a/ (a+c) x100 = 53/57x 100 = 92.98%

Specificity = d/ (b+d) x100 = 49/49x 100 = 100%

Predictive value of positive test = a/ (a+b) x100 = 53/53x 100 = 100%

Predictive value of negative test = d/ (c+d) x100 = 49/53 x 100 = 92.45%

Percentage of false negative = c/ (a+c) x100 = 4/57 x 100 = 7.01%

Percentage of false positive = b/ (b+d) x100 = 0/49 x 100 = 0%

B) Comparision of TB-PCR with reference to culture

	Test and result	Culture		Total
		Positive	Negative	
PCR	Positive	55 (a)	4(b)	59 (a+b)
	Negative	2 (c)	45 (d)	47 (c+d)
	Total	57 (a+c)	49 (b+d)	106 (a+b+c+d)

Sensitivity= a/ (a+c) x100 =55/57 x 100 = 96.49%

Specificity = d/ (b+d) x100 = 45/49 x 100 = 91.83%

Predictive value of positive test = a/ (a+b) x100 = 55/59 x 100 = 93.22%

Predictive value of negative test = d/ (c+d) x100 = 45/47 x 100 = 95.74%

Percentage of false negative = c/ (a+c) x100 = 2/57 x 100 = 3.50%

Percentage of false positive = b/ (b+d) x100 = 4/49 x 100 = 8.16%

C) Comparison of LAMP with reference to culture

	Test and result	Culture		Total
		Positive	Negative	
LAMP	Positive	55 (a)	1(b)	56 (a+b)
	Negative	2 (c)	48 (d)	50 (c+d)
	Total	57 (a+c)	49 (b+d)	106 (a+b+c+d)

Sensitivity= a/ (a+c) x100 = 55/57x 100 = 96.49%

Specificity = d/ (b+d) x100 = 48/49x 100 = 97.95%

Predictive value of positive test = a/ (a+b) x100 = 55/56x 100 = 98.21%

Predictive value of negative test = d/ (c+d) x100 = 48/50 x 100 = 96%

Percentage of false negative = c/ (a+c) x100 = 2/57 x 100 = 3.50%

Percentage of false positive = b/ (b+d) x100 = 1/49 x 100 = 2.04%

D) Comparison of LAMP with reference to PCR

	Test and result	PCR		Total
		Positive	Negative	
LAMP	Positive	56 (a)	0 (b)	56 (a+b)
	Negative	3 (c)	47 (d)	50 (c+d)
	Total	59 (a+c)	47 (b+d)	106 (a+b+c+d)

Sensitivity= a/ (a+c) x100 = 56/59x 100 = 94.9%

Specificity = d/ (b+d) x100 = 47/47x 100 = 100%

Predictive value of positive test = a/ (a+b) x100 = 56/56x 100 = 100%

Predictive value of negative test = d/ (c+d) x100 = 47/50 x 100 = 94%

Percentage of false negative = c/ (a+c) x100 = 3/59 x 100 = 5.08%

Percentage of false positive = b/ (b+d) x100 = 0/47 x 100 = 0%

Appendix XII: Classification of *Mycobacteria*

Mycobacterial 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 favorable 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 rifampicin Usually pathogenic causes "Swimming pool granuloma"; only in skin lesions (never in sputum); susceptibility to streptomycin. Nonpathogenic; 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. intracellulare* *X. xenopi* *M. ulcerans*	Both species are usually pathogenic; most stains are resistance to anti tuberculosis drugs. Commonly nonpathogenic; susceptible to isoniazid and streptomycin Usually 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 anti tuberculosis drugs.

Source: Pelczar *et al.*, 1993.

Appendix XIII: Treatment regimens for tuberculosis

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

Essential anti-TB drugs (Abbreviation)	Mode of action	Recommended dose (mg/kg) intermittent		
		Daily	3x/week	2x/weeek[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.

Possible alternative treatment regimens for each treatment category

TB treatment category	TB patients	Alternative TB treatment regimens	
		Initial phase (daily or 3 times per week)	Continuation phase
I	New smear-positive PTB; New smear-negative PTB with extensive parenchymal involvement; new cases of severe forms of extra-pulmonary TB	2 EHRZ (SHRZ) 2 EHRZ (SHRZ) 2 EHRZ (SHRZ)	6HE 4HR $4H_3R_3$
II	Sputum smear- positive: relapse; Treatment failure; Treatment after interruption.	2 SHRZE/1HRZE 2 SHRZE/1HRZE	$5\ H_3\ R_3\ E_3$ 5 HRE
III	New smear-negative PTB (other than in category I); new less severe forms of extra-pulmonary TB	2 HRZ 2 HRZ 2 HRZ	6HE 4 HR 4 H3R3
IV	Chronic case (still sputum-positive after supervised re-treatment)	Not applicable (Refer to WHO guidelines for use of second-line drugs in specialized centers)	

Source: Maher *et al*., 1997

There is a standard code for TB treatment regimens. Each anti-TB drugs has an abbreviation (shown in above table). A regimen consists of 2 phases. The number before a phase is the duration of that phase in months. A number in subscript (e.g. 3) after a letter is the number of doses of that drug per week. If there is no number in subscript after a letter, then treatment with that drug is daily. An alternative drug (or drugs) appears as a letter (or letters) in brackets.

Appendix XIV: Results of total samples with culture, LAMP and PCR

A) Results 53 smear positive samples with culture, LAMP and PCR (Group A)

S.N.	Sample Code	Flourochrome staining (Microscopy)	Culture on Ogawa medium	LAMP with six primers	PCR with two primers
1.	399B	Positive	Positive	Positive	Positive
2.	347B	Positive	Positive	Positive	Positive
3.	320 B	Positive	Positive	Positive	Positive
4.	361 B	Positive	Positive	Positive	Positive
5.	360B	Positive	Positive	Positive	Positive
6.	344B	Positive	Positive	Positive	Positive
7.	128B	Positive	Positive	Positive	Positive
8.	150B	Positive	Positive	Positive	Positive
9.	5586B	Positive	Positive	Positive	Positive
10.	5567B	Positive	Positive	Positive	Positive
11.	195B	Positive	Positive	Positive	Positive
12.	257B	Positive	Positive	Positive	Positive
13.	233B	Positive	Positive	Positive	Positive
14.	239B	Positive	Positive	Positive	Positive
15.	451B	Positive	Positive	Positive	Positive
16.	471B	Positive	Positive	Positive	Positive
17.	473B	Positive	Positive	Positive	Positive
18.	500B	Positive	Positive	Positive	Positive
19.	515B	Positive	Positive	Positive	Positive
20.	522B	Positive	Positive	Positive	Positive
21.	536B	Positive	Positive	Positive	Positive
22.	482B	Positive	Positive	Positive	Positive
23.	570B	Positive	Positive	Positive	Positive
24.	603B	Positive	Positive	Positive	Positive
25.	605B	Positive	Positive	Positive	Positive
26.	606B	Positive	Positive	Positive	Positive
27.	612B	Positive	Positive	Positive	Positive

28.	618B	Positive	Positive	Positive	Positive
29.	623B	Positive	Positive	Positive	Positive
30.	666B	Positive	Positive	Positive	Positive
31.	703B	Positive	Positive	Positive	Positive
32.	706B	Positive	Positive	Positive	Positive
33.	445B	Positive	Positive	Positive	Positive
34.	452B	Positive	Positive	Positive	Positive
35.	430B	Positive	Positive	Positive	Positive
36.	287B	Positive	Positive	Positive	Positive
37.	288B	Positive	Positive	Positive	Positive
38.	291B	Positive	Positive	Positive	Positive
39.	340B	Positive	Positive	Positive	Positive
40.	338B	Positive	Positive	Positive	Positive
41.	206B	Positive	Positive	Positive	Positive
42.	573B	Positive	Positive	Positive	Positive
43.	845B	Positive	Positive	Positive	Positive
44.	809B	Positive	Positive	Positive	Positive
45.	824B	Positive	Positive	Negative	Negative
46.	898B	Positive	Positive	Positive	Positive
47.	912B	Positive	Positive	Positive	Positive
48.	908B	Positive	Positive	Positive	Positive
49.	904B	Positive	Positive	Positive	Positive
50.	2906B	Positive	Positive	Negative	Negative
51.	2945B	Positive	Positive	Positive	Positive
52.	2908B	Positive	Positive	Positive	Positive
53.	2888B	Positive	Positive	Positive	Positive

B) Results 53 smear Negative samples with culture, LAMP and PCR (Group B)

S.N.	Sample Code	Flourochrome staining (Microscopy)	Culture on Ogawa medium	LAMP with six primers	PCR with two primers
1.	412B	Negative	Negative	Negative	Negative
2.	358B	Negative	Negative	Negative	Negative

3.	365B	Negative	Negative	Negative	Negative
4.	353B	Negative	Negative	Negative	Negative
5.	136B	Negative	Negative	Negative	Negative
6.	160B	Negative	Negative	Negative	Negative
7.	154B	Negative	Negative	Negative	Negative
8.	5553B	Negative	Negative	Negative	Negative
9.	5570B	Negative	Positive	Positive	Positive
10.	109B	Negative	Negative	Negative	Negative
11.	5566B	Negative	Negative	Negative	Negative
12.	159B	Negative	Negative	Negative	Negative
13.	168B	Negative	Negative	Negative	Negative
14.	181B	Negative	Negative	Negative	Negative
15.	186B	Negative	Negative	Negative	Negative
16.	198B	Negative	Negative	Negative	Negative
17.	205B	Negative	Negative	Negative	Negative
18.	207B	Negative	Negative	Negative	Negative
19.	254B	Negative	Negative	Negative	Negative
20.	258B	Negative	Positive	Positive	Positive
21.	267B	Negative	Negative	Negative	Negative
22.	463B	Negative	Negative	Negative	Negative
23.	425B	Negative	Negative	Negative	Negative
24.	453B	Negative	Negative	Negative	Positive
25.	444B	Negative	Positive	Positive	Positive
26.	479B	Negative	Negative	Negative	Negative
27.	482B	Negative	Negative	Negative	Negative
28.	486B	Negative	Negative	Negative	Negative
29.	501B	Negative	Negative	Negative	Negative

30.	502B	Negative	Negative	Negative	Negative
31.	509B	Negative	Negative	Negative	Negative
32.	513B	Negative	Negative	Negative	Negative
33.	525B	Negative	Negative	Negative	Negative
34.	508B	Negative	Negative	Negative	Negative
35.	533B	Negative	Negative	Negative	Negative
36.	588B	Negative	Negative	Negative	Negative
37.	601B	Negative	Negative	Negative	Negative
38.	455B	Negative	Negative	Negative	Negative
39.	389B	Negative	Negative	Negative	Negative
40.	427B	Negative	Negative	Negative	Negative
41.	431B	Negative	Negative	Negative	Negative
42.	378B	Negative	Negative	Negative	Negative
43.	306B	Negative	Negative	Negative	Negative
44.	345B	Negative	Negative	Negative	Negative
45.	313B	Negative	Positive	Positive	Positive
46.	323B	Negative	Negative	Positive	Positive
47.	333B	Negative	Negative	Negative	Negative
48.	335B	Negative	Negative	Negative	Positive
49.	275B	Negative	Negative	Negative	Negative
50.	283B	Negative	Negative	Negative	Negative
51.	393B	Negative	Negative	Negative	Positive
52.	398B	Negative	Negative	Negative	Negative
53.	800B	Negative	Negative	Negative	Negative

Appendix XV: Questionnaire for data collection

Name : Pt. No. :

Age : Lab No. :

Sex :

Address :

Contact No. :

1. Symptoms :

i) Fever :- Yes No ii) Chest Pain :- Yes No

Duration : Duration :

iii) Weight loss :- Yes No iv) Cough :- Yes No

Duration : Duration :

v) Haemoptysis :- Yes No

Duration :

2. Do you have habit of smoking ? Yes No

Duration :

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

Duration :

4. What your family history of TB ? Present Absent

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

6. Did you have TB in past ? Yes No

7. X-ray Report :- Positive Negative Not Done

8. Blood Report :-

HIV :- Positive Negative Not Done

HB_S Ag :- Positive Negative Not Done

9. Date of Collection :

10. Time of Collection :-

QUESTIONNAIRE FOR DATA COLLECTION

Name : Pt. No. :

Age : Lab No. :

Sex :

Address :

Contact No. :

1. Symptoms :

i) Fever :- Yes No ii) Chest Pain :- Yes No

Duration : Duration :

iii) Weight loss :- Yes No iv) Cough :- Yes No

Duration : Duration :

v) Haemoptysis :- Yes No

Duration :

2. Do you have habit of smoking ? Yes No

Duration :

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

Duration :

4. What your family history of TB ? Present Absent

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

6. Did you have TB in past ? Yes No

7. X-ray Report :- Positive Negative Not Done

8. Blood Report :-

HIV :- Positive Negative Not Done

HB_S Ag :- Positive Negative Not Done

ESR :-

Mantoux Text Positive Negative

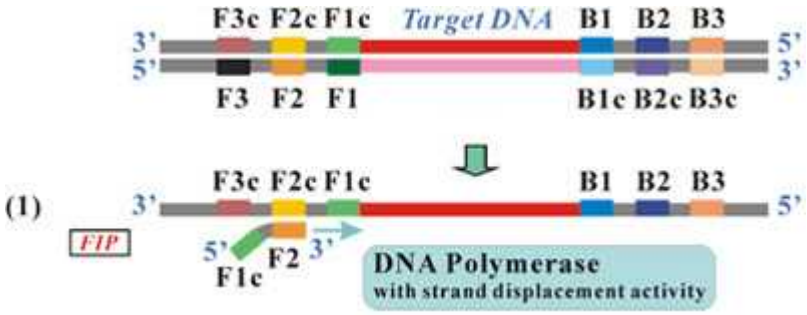
Size :-

9. Date of Collection :

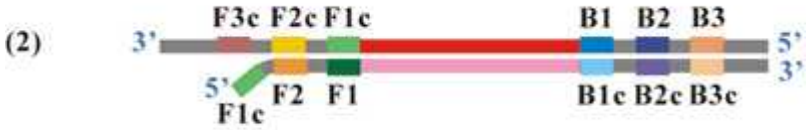
10. Time of Collection :-

Figure 1: Basic principle of LAMP

STEP1



STEP2



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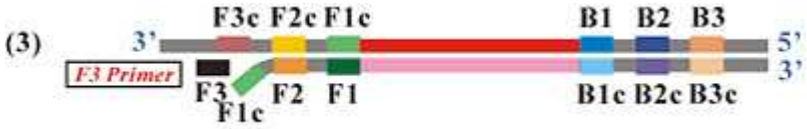

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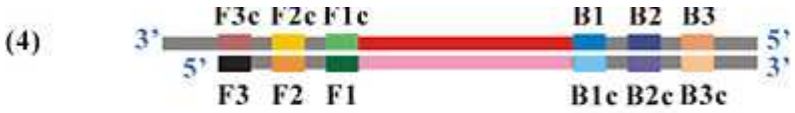
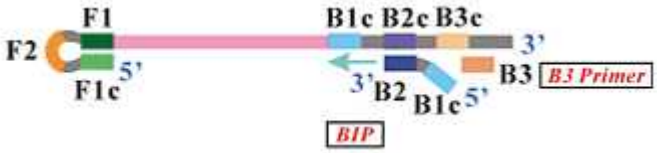


STEP5

STEP6

(6)



STEP7

(7)

STEP8

(8)

Basic principle 8-11(Cycling Amplification step).