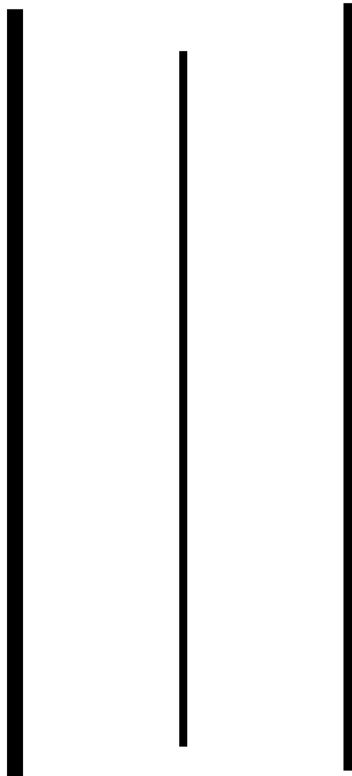
Loop Mediated Isothermal Amplification

For The Direct Detection of Human Pulmonary Infection with

Mycobacterium Tuberculosis, Mycobacterium Avium Complex and

Mycobacterium Kansasii from Sputum



Submitted to

Nepal Health Research Council Ramshahpath, Kathmandu

Submitted by:

Dr. Basu Dev Pandey

Principle Investigator

Date: 7th May 9, 2009

To the Member Secretary,

Nepal Health Research Council

Ramshahpath, Kathmandu, Nepal

Re: Submission of final report

Dear Sir,

As our contract for the experienced scientist research grant for the year 2008/ 09 with the NHRC, the study entitled "Loop mediated Isothermal Amplification For The Direct Detection of Human Pulmonary Infection with Mycobacterium *Tuberculosis*, Mycobacterium *Avium* Complex and Mycobacterium *Kansasii* from Sputum" has completed successfully. The final report of this study is submitted for your kind attention and necessary action.

Thank you,

Sincerely yours,

Dr. Basu Dev Pandey

Principle Investigator

ACKNOWLEDGEMENT

The PI is grateful to all the doctors, nurses, laboratory staffs of the participating hospitals including Sukraraj Tropical and Infectious Diseases Hospital, Nepal Dr. Bhawana Shrestah and Bhagwan Maharjan, Lab Incharge of German- Nepal Tuberculosis Project (GENETUP), Kalimati for their co-operation for the collection of samples. I also thanks to all the staff of Everest International Clinic and Research Center (EICRC) for their technical help and providing the laboratory facility for the study. I would also like thanks to Mr Balaram Adhikari, Microbiologist, National Public Health Laboratory, Mr Ajya Poudel, Microbiologist, Kathmandu Medical College, Kiran Pandey from EICRC for their coordination, technical supervision of overall activities in the laboratory work and Master student Manju Tamang, Central Department Tribhuban University, Kathmandu, Nepal for her hard work in collecting samples and laboratory work. Finally, I am very much grateful to the patients for their participation and NHRC for providing research grants for the study.

Dr. Basu Dev Pandey

Principle Investigator

ABSTRACT

Introduction: Rapid species identification and proper use of drugs are key requirements for the effective treatment and case management of tuberculosis. The development and evaluation of new diagnostic technique, which can diagnose causative agent in simple and rapid way, is the necessity of this century. Loop-Mediated Isothermal Amplification (LAMP) provides new possibilities of above requirements for direct detection of *M. tuberculosis, M. avium* complex and *M. kansasii* in sputum samples.

Objectives: To diagnose Mycobacaterium Tuberculosis early and accurately and to identify the prevalence of atypical mycobactera in Nepal. The study further intended to initiate LAMP as a diagnostic tools in hospital laboratories of Nepal.

Materials and Methods: This study was carried out 130 sputum samples from suspected pulmonary tuberculosis patients were included in this study. All these samples were processed for flurochrome staining and were subjected to culture and LAMP. Thus sputum specimens were included in this study to compare them with microscopy, culture and LAMP.

Results: Among them 50(38.46%) were found to be positive by flurochrome staining, culture and LAMP. Similarly 48(36.92%) samples were negative by all diagnostic methods. 1(0.77%) microscopy and culture positive sample was negative by LAMP. Similarly 3(2.31%) microscopy and LAMP positive cases were negative by culture. 3(2.31%) culture positive cases were negative by both microscopy and LAMP. Eight (6.16%) culture negative cases were positive by LAMP where as 17(13.07%) microscopy negative samples were positive by culture and LAMP. Out of 78(100%) total LAMP positive cases, 76(97.44%) were positive for *M. tuberculosis* and remaining 2 (2.56%) were positive for *M. intracellular*. While comparing LAMP results with gold standard culture, 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 94.36%, 81.36%, 85.90%, 92.31%, 5.63% and 18.64% respectively. Similarly, LAMP had sensitivity 98.14% and specificity 67.11% while compare with microscopy.

Discussion and Conclusion: LAMP is highly sensitive and specific molecular technique, which can be used effectively for the diagnosis of clinically, microscopically, and culturally confusing cases thus facilitating the effective treatment and case management of tuberculosis and other atypical mycobacterial infection. Due to its easy operation and rapid amplification efficiency, it can be used in well-equipped laboratories for clinical use if sample preparation, nucleic acid extraction and cross-contamination controls are addressed.

Key words: *M. tuberculosis, M avium* complex*, M. kansasii*, LAMP

LIST OF ABREVIATIONS

AFB	:	Acid fast bacilli
BCG	:	Calmette-Guerin Bacilli
d ATP	:	2'- deoxyadenosine 5'-triphosphate
d TTP	:	2'-deoxythymine 5'-triphosphate
d CTP	:	2'-deoxycytidine 5'-triphosphate
d GTP	:	2'-deoxyguanosine 5'-triphosphate
d NTPs	:	Deoxyrionucleoside triphosphates
DNA	:	Deoxyribonucleic acid
DOH	:	Department of Health Services, Nepal
FD	:	Fluorescence Dye
HIV	:	Human Immuno-Deficiency Virus
IFN	:	Interferon
LAMP	:	Loop-Mediated Isothermal Amplification
L-J	:	Lowenstein-jensen Medium
MAC	:	Mycobacterium avium complex
MAC-PD	:	Mycobacterium avium complex-Pulmonary Disease
MAV	:	Mycobacterium avium
MIN	:	Mycobacterium intracellular
MK	:	Mycobacterium kansasii
MK-PD	:	Mycobacterium kansasii- Pulmonary Disease
MTB	:	Mycobacterium tuberculosis bacilli
MOTT	:	Mycobacteria Other Than Tuberculosis
NAA	:	Nucleic Acid Amplification
NALC	:	N-acetyl-L-cysteine
NTC	:	National Tuberculosis Center
NTP	:	National Tuberculosis Programme
NTM	:	Non tuberculosis Mycobacteria
NK	:	Natural killer cell
OD	:	Optical Density

PCR	:	Polymerase Chain Reaction
PTB	:	Pulmonary Tuberculosis
RFLP	:	Restriction fragment length polymorphism
rpm	:	Revolution Per Minute
TB	:	Tuberculosis
WHO	:	World Health Organization
Z-N	:	Ziehl-Neelsen

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CHAPTER I: INTRODUCTION

1. Background and significance of the study:

TB is one of the major health problems in Nepal. Each year more than 45,000 of people are infected by this disease. Although the introduction of Directly Observed Therapy Short course (DOTS) methods has reduced the number of deaths, over 6000 in Nepal continue to die of TB every year (NTC 2004). Diagnosis of TB depends on the clinical evaluation of the patients including chest radiography, Tuberculin skin test and bacteriological test. Identification of Mycobacterial isolates in the species level is a trouble some job for clinical laboratories. The conventional biochemical tests for identification of Mycobacterial species are slow and time-consuming because of the slow growth of *Mycobacterium* on culture media. Similarly microscopy method is unable to distinguish within the mycobacterium genus. In contrast, bacteriological testing using bacterial cultures are considered the most accurate test due to its high sensitivity and specificity. However, this technique is both labour-intensive and time-consuming, requiring 6-8 weeks to achieve maximum sensitivity. An additional technique, nucleic acid amplification assay (NAA), allows the rapid, sensitive and specific detection of *M. tuberculosis* in sputum samples by amplifying and detection specific nucleic acid sequences. In addition to the widely used PCR, several other methods of nucleic acid amplification have been used for *M. tuberculosis* detection. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method which enables the detection of trace amounts of DNA under isothermal conditions, namely at 64^0C. LAMP showing high amplification efficiency has been used for the diagnosis of several diseases including Tuberculosis.

In Nepal, 45 % of the total population is infected with TB, out of which 60 % are adult. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. On the other hand TB/HIV co-infection has emerged a serious issue in the diagnosis and treatment of TB. The targets of diagnosing 70 % of new infectious cases and curing 85 percent of these patients we will prevent 50,000 deaths over the next five years. However, there is no information on remaining 15

percent of the cases not cured by DOTS. We are concerned that they might be cases of MDR, XDR or atypical mycobacteria not responding with the DOTS therapy. Environmental (Nontuberculosis) mycobacteria have been documented to be involved in human pulmonary infections in both developed and developing countries. Incidence rates of between 2-20% for atypical mycobacteriosis have been reported from various parts of the world. In the past we evaluated mycobacterium tuberculosis sputum samples of Nepalese patients and established Loop Mediated Nucleic Acid Amplification (LAMP) system in Nepal. In this study, we are evaluating the presence of atypical mybacterium in the sputum samples.

1.2 Statement of the Problem

TB is a major public health problem in Nepal. About 45 percent of the total population is infected with TB, out of which 60 percent are adult. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease (Annual Report, DOH, Nepal). Introduction of treatment by Directly Observed Treatment Short course (DOTS) has already reduced the numbers of deaths; however 6000 people continue to die every year from this disease. The targets of diagnosing 70 percent of new infectious cases and curing 85 percent of these patients we will prevent 50,000 deaths over the next five years. However, we don't know the remaining 15 percent of the cases not cured by DOTS. We are concern that they might be cases of MDR or Atypical TB not responding with the DOTS therapy.

The accurate diagnosis and treatment of classical tuberculosis is concerned since it give opportunity for the early treatment eventually prevent the transmission of the diseases. The conventional methods used for the diagnosis of TB are clinical symptoms, chest X-ray, sputum smear microscopy, tuberculin test and in vitro culture methods for tuberculosis Clinically and radiologically, the pulmonary disease produced by environmental strains is indistinguishable from classical pulmonary tuberculosis, whereas the two diseases present fundamental differences in their epidemiology, management and prognosis. It is therefore important that clinicians and bacteriologists appreciate the fact that not all Acid fast bacilli found in sputum are *Mycobacterium tuberculosis*. Therefore rapid and easy diagnosis of mycobacteria in

the species level is the necessity for the well management of tuberculosis and tuberculosis control programme in Nepal.

1. 3. Objectives:

a) General

- To use LAMP for the direct detection of Mycobacterium tuberculosis, Mycobacterium avium complex and Mycobacterium kansasii from sputum to support clinician for early treatment

b) Specific:

- To diagnose Mycobacaterium Tuberculosis early and accurately.
- To identify the prevalence of atypical mycobactera in Nepal.
- To initiate LAMP as a diagnostic tools in hospital laboratories of Nepal

1.4 Preparation of study:

The research team was formed for the clinical information, data collection, filling of questioners, sample collection and laboratory assays. The research plan was formed under the co-ordination of PI. The other investigators in this study involved are Mr Balaram Adhikari, Mr Ajay Poudel, Manju Tamang and Kiran Pandey. All the questioners, reagents and equipment including the sample carrying boxes needed for the study was prepared. Study sites were identified as mentioned in the proposal. An orientation session was conducted to explain the aim of the study, appropriate collection techniques of samples before the study. The importance of appropriate storage of serum samples and transportation was explained to the team.

1.5 Data collection from the patient visited to the hospital and using prepared questioner's sheet.

Prepared questioners were used to collect clinical and other relevant information of the patients. All the information's including age, sex, literacy status, occupation were asked after taking the informed consent. Clinical history including duration of fever, cough and chest pain or haemoptyis was recorded. For the new patients suspected of pulmonary tuberculosis, three early morning

sputum samples were taken from the patient visited to the hospital. All the samples were collected in leak proof, wide mouth and transparent, sterile and stopper plastic container. 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 processed for the sputum microscopic examination. Digestion, Decontamination and Concentration were performed as per standard methodology.

CHAPTER II: LITERATURE REVIEW

TB is one of the major health problem with 5.1 million new cases and 1.6 million deaths each year worldwide (WHO, 2007). In Nepal, 45 percent of the total population is infected with TB, out of which 60 percent are adult. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease (Annual Report, DOH, Nepal). It is a common and deadly infectious disease caused by the *Mycobacterium tuberculosis,* affects the lungs but can also affect the central nervous system the lymphatic system, the circulatory system, the genitourinary system, joints and even the skin. The commonest sites of infection are: lymph glands and abscesses particularly around the neck, bones and joints. The spine is affected in about half such cases. Meningitis, which may be rapidly fatal if not, treated in time

Chronic pulmonary disease is the most common localized clinical manifestation of NTM. *Mycobacterium avium* complex (MAC), followed by *M. kansasii*, are the most frequent pathogens causing lung disease. Other atypical pathogens occasionally causing pulmonary disease include *M. abscessus, M. fortuitum*, *M.szulgai, M.simiae, M. xenopi, M.malmoense*, *M. celatum*, *.M. asiatium and M. shimodii*. The patients with chronic lung disease due to NTM are generally older adults and children also develop this form of NTM disease. The interpretation of NTM in the sputum of HIV-positive patients presents a particular problem, as these patients are frequently infected with NTM without evidence of pulmonary disease.

Organisms belonging to the genus mycobacterium are very thin, rod-shaped (0.2-0.4×2 to 10µm) and non motile. They are aerobic, non capsulated and resist decolorization by acidified alcohol (*Forbes et al., 2000*). The genus belongs to the mycobacteraceae family, Actinomycetales order and Actinomcetes class. The high G+C content of the DNA of Mycobacterium species is 61 to 70 mol%. Mycobacterium species have an unusual cell wall structure that contains N-glycolylmuramic acid instead of N-acetylmuramic acid and has very high lipid content. Because of this cell wall structure, mycobacteria are difficult to stain with commonly used basic aniline dyes. They grow more slowly and organisms tend to clump, so that nutrients are not easily allowed into the cell. Growth is slow with colonies become visible in 2 to 60 days at optimum temperature.

Clinically Mycobacteria are divided into two major groups based on the fundamental differences in epidemiology and association with disease: those associated with *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum)* and others *non-tuberculous mycobacteria* (NTM).

M. tuberculosis is non-sporing, non capsulated, straight and slightly curved rod measuring 1-4×0.2- 0.5 µm). Tubercle bacilli are aerobes, grow slowly with generation time 12-24 hours, colonies usually appears in 2-3 weeks and may sometimes requires 8 weeks incubation, optimum temperature 37°C and pH 6.4-7.0 The tubercle bacilli grow on Lowenstein-Jensen (LJ) medium is widely used. The colonies of *M. tuberculosis* on LJ medium are dry, rough, and creamy or buff colored.

MAC are slow growing, non-sporing, non-capsulated and indistinguishable morphologically from those of *M. tuberculosis*. They are referred to as non-photochromogenic as they do not produce pigment colonies on incubation either in light or dark. Typical culture of MAC on L-J medium is smooth, yellow colored and they are non-adherent (they do not produce "cord factor"). *M. kansasii* is also a non-sporing, non-capsulated, straight rod. Morphologically, it is similar to that of *M. tuberculosis*. It belongs to a group of Mycobacteria referred to as photochromogenic because they produce pigmented colonies when grown in presence of white light but not when incubated in the dark. The colonies of *M. kansasii* on L-J medium are smooth, yellow colored and they are non-adherent.

TB is transmitted from person to person mainly by droplet infection and droplet nuclei containing viable virulent organism generated by sputum positive patients with pulmonary tuberculosis (PTB) during coughing, sneezing and vocalizing. The incubation period generally ranges form 3 to 6 weeks and some times up to months or years. Epidemiologic studies, skin test surveys and more recently DNA fingerprinting studies suggest that person-to-person transmission of pulmonary infection due to *M. avium, M. intracellular* and *M. kansassii* and other NTM are rare. Most persons are infected by environmental NTM.

Therapy generally consists of 6 to 9 month course of Isoniazid, rifampicin, streptomycin, thiocetazone, pyrazinamide and ethambutol. Most first-line anti

tuberculosis drugs have 10-100 times less in vitro activity against *M. avium* complex isolates than against *M. tuberculosis* due to the lipophilic cell wall of *M. avium* complex, which prevents drug penetration. Treatment *of M. avium complex* pulmonary disease requires a regimen of daily clarithromycin twice or azithromycin, rifampin or rifabutin, and ethambutol for therapy of adults for 1 year. Treatment of disseminated *M avium* complex disease in adults should include daily clarithromycin or azithromycin, plus ethambutol. A regimen of daily isoniazid, rifampin, and ethambutol for 18 month with a minimum of 12 month culture negativity is recommended for pulmonary disease in adults caused by *M. Kansasii*. The timely identification of person infected with tuberculosis and their rapid laboratory conformation of tuberculosis are two key ingredients of effective public health measure for the control of the TB.

Diagnosis of active disease includes clinical suspicion, chest radiographs, staining for acid-fast bacilli, and culture for mycobacterium, more recently, and nucleic acid amplification assay. Acid-fast microscopy is done for the rapid identification of patients with mycobacterium infection, utilizing 'acid fast' property of mycobacterium. Presently; two types of acid-fast stains are used in laboratories. One is carbol fuchsin (Ziehl-Neelsen (Zn) or Kinyoun staining methods) and the other is flurochrome (usually auramine or aurmine-rhodamine). In the carbol fuchsin procedure, acid-fast organisms appear red against a blue background, while in the flurochrome procedures; the acid-fast organisms appear as fluorescent rods, yellow to orange against a pale yellow or orange background .Whenever disease is suspected, three specimens must be collected for examination by microscopy that increases the predictive value of positivity of smear microscopy, reaching almost that of culture. This method detects 5,000-10,000 bacteria per ml with sensitivity range between 46-78%.

Until now, culture is taken as a gold standard method for diagnosis of mycobacteria in the specimen. A combination of different culture media is required to optimize the recovery of mycobacteria from culture; at least one solid medium in addition to a liquid medium should be used (Forbes et al., 2002).There are two types of culture media: solid media, which include egg-based media (Lowenstein-Jensen and Ogawa medium) and agar basedmedia. Cultures should be inoculated onto one or more solid media and into a liquid medium. At least three sputum cultures should be used for the

initial evaluation. Lowenstein-Jensen is an excellent medium for recovery of *M. tuberculosis*, but is generally inferior to middlebrook agar as an all-purpose medium for both *M. tuberculosis* and *NTM*. Cultures are examined weekly for growth, contaminated cultures are discarded and reported as "contaminated, unable to detect presence of mycobacteria". Most isolates appear between 3 and 6 weeks; a few isolates appear after 7 or 8 weeks of incubation. Sputum culture is a highly sensitive diagnostic method that permits detection of a minimum of 10 to 100 viable bacilli per ml of cultured material. Culture is more sensitive for the detection of mycobacteria than acid-fast microscopy. It provides drug susceptibility tests and genotyping of particular cultured NTM can be used for epidemiologic purposes and to rule out cross contamination. *M. tuberculosis* is an obligate aerobe, grows optimally at 37^0C and PH 6.4-7.0. It is a slow growing organism with generation time of 14-15 hours; on solid medium *M. tuberculosis* forms a dry, rough, raised, irregular, white colony with wrinkled surface.

MAC also are obligate aerobe, grows optimally at 37^0c and slow growing organism. On solid media (Ogawa and L-J). MAC produces smooth and non-adherent colonies. As they do not produce pigment, colonies are colorless. Wallenstein's medium is an excellent medium for the recovery of NTM, particularly MAC. On solid media, *M. kansasii* produces same colonies as MAC but as a photochromogenic organism it produce pigment and the colonies are yellow in colored. Pigment is produce when grown in presence of white light but not when incubated in dark.

Molecular methods offer many advantages over conventional methods of identification for the diagnosis nontuberculous mycobacterium. Probes are widely used in clinical laboratories for the identification of the most common mycobacterial species as a routine method for species identification. In addition to the widely used PCR-based detection, several amplification methods like nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR), transcription-mediated amplification (TMA), oligonucleotide ligation amplification and strand displacement amplification (SDA).

Loop-mediated isothermal amplification (LAMP) is novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under

isothermal conditions. It starts with the mixing of the buffer primers, DNA lysates and DNA polymerase in tube, and then the mixture is incubated at 64°C for 1 hour. Visual judgment eliminates the need for any laboratories and time consuming post amplification operations such as hybridization and electrophoresis as well as the need for special equipment. Therefore, LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of infectious disease both in well-equipped laboratories and in field situations. The addition of loop primers shortens reaction time for amplification by one third to one half by hybridizing to the stem-loops, except for the loops that are hybridized by the inner primers (Nagamine et al., 2002).

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

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

Several investigators utilized LAMP for the diagnosis of Infectious diseases. Iwamoto et al. (2003) used LAMP for detection of *M. tuberculosis* complex, *M. avium* and *M. intracellular* directly from sputum specimens as well as for detection of culture isolates grown in a liquid medium or on a solid medium (Ogawa's medium). Species-specific primers were designed by targeting the *gyr*B gene, and their specificities were validated on 24 mycobacterial species and 7 nonmycobacterial species. The 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 μl of the DNA lysates, it showed a performance comparable with that of the Amplicor assay, which required 50 μl 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.

Saito et al. (2005) developed and evaluated LAMP assay for the rapid detection of *Mycoplasma pneumoniae*. The assay specifically amplified only *M. pneumoniae* sequences, and no cross-reactivity was observed for other *Mycoplasma species* or rspiratory bacterial species. The detection limit for this assay was found to be 2×10^2 copies, corresponding to 2-20 color changing units of *M. pneumoniae* in 1 h, as observed in a real-time turbidimeter and electrophoretic analysis. The accuracy of the LAMP reaction was confirmed by restriction endonuclease analysis as well as direct sequencing of the amplified product. The assay was applied to 95-nasopharyngeal swab samples collected from patients or from healthy individuals, and compared to a real-time PCR assay in house. A concordance of 100% was observed between the two assays.

Nagamine et al. (2002) 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 μl of serum as template, LAMP amplification was able to detect signals after 25-35 min in five individuals. This result revealed that the presence of HBV virus can be detected within 1 h from a non denatured sample. In separate experiments, however, LAMP was performed successfully without heat denaturation for template DNAs, such as DNA, pBluescript II, and M13 mp 18 vector DNA, and human genomic DNA (SRY gene on chromosome Y), including commercially available material. Some of the double-stranded DNA seemed to become single-stranded at high temperatures in the presence of high concentrations of betaine, a reagent that facilitates DNA strand separation because it stabilizes DNA. Because there is no necessity for heat denaturation of the template DNAs, LAMP could be used more easily and rapidly in clinical medicine.

Nagamine et al. (2002) has developed a method to isolate single-stranded DNA fragments from LAMP products that are stem-loop DNAs with several inverted repeats of the target DNA. This method required the TspRI restriction enzyme, a primer hybridized to the 3' overhanging sequence at its cleavage site, and a DNA polymerase with strand displacement activity. The LAMP products were digested with TspRI and were then extended using the primer, producing the strand -specific DNA fragments. All

processes, from LAMP reaction to primer extension, were carried out at the same temperature. So, the use of strand-specific DNA would be conducive for detection by hybridization technique such as DNA microarrays.

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

Notami et al. (2000) developed LAMP in order to demonstrate the mechanism, the efficiency, and ease of use of LAMP. They chose M13mp18 DNA as a model target DNA, and prepared four primers that met the LAMP requirement. The LAMP produced many bands of different sizes from 300 bp to the loading well. Production of bands depended on the presence of inner primers, the template and DNA polymerase. When the products were analyzed by alkaline agarose gel electrophoresis, smeared DNA between bands and at the well was shifted to bands of < 10bp. Endo et al. (2004) detected the species-specific gp43 gene of *Paracoccidiolides brasiliensis* by LAMP in 22 clinical and seven armadillo-derived isolates. The amplified DNA appeared as a ladder with a specific bandings pattern. They were also able to obtain positive results from DNA extracted from a paraffin-embedded tissue sample of *paracoccidioidonycosis,* suggesting that this method may achieve clinical application in the near future.

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

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

Kaneko et al. (2005) used a LAMP assay for the detection of herpes simplex virus types I (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus (VZV). To evaluate the application of the LAMP assay for clinical diagnosis, they tested clinical samples from 40 genital herpes patients and 20 ocular herpes patients. With the LAMP assay, 41 samples with DNA extraction and 26 direct samples without DNA extraction were identified as positive for HSV-1 or HSV-2, although 37 samples with DNA extraction and just one without DNA extraction were positive by a PCR assay. Thus, the LAMP assay was less influenced than the PCR assay by the presence of inhibitory substances in clinical samples. These observations indicate that the LAMP assay is very useful for the diagnosis of HSV-1, HSV-2, and VZV infections.

In Nepal, Pandey et al 2008, evaluated LAMP for the diagnosis of Mycobacterium for sputum samples of Nepalese patients. In this study, one hundred microscopy positive and another100 negative sputum samples were used to evaluate the assay. They found the test is highly sensitive and specific equivalent to bacterial cultures.

CHAPTER III: MATERIALS AND METHODS

3.1 Material

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

Primers

F3: 5' CTGGCTCAGGACGAACG 3'

FIP: 5' CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT 3'

FL: 5' GTTCGCCACTCGAGTATCTCCG 3'

BL: 5' GAAACTGGGTCTAATACCGG 3'

BIP: 5' TCGGGATAAGCCTGGACCACAAGACATGCATCCCCT 3'

B3: 5' GCTCATCCCACACCGC 3'

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

SYBER Green I – Fluorescence dye

Beatain - N,N,N-trimethylglycine

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

3.2 Methods

3.2.1 Study area

This study was carried out in collaboration with Sukraraj Tropical and Infectious Disease Hospital, National TB Reference Lab of German-Nepal Tuberculosis Project (GENETUP) and Everest International Clinic and Research Center (EICRC), Kathmandu Nepal

3.2.2 Sample Collection

Sputum samples were collected from the suspected pulmonary TB patients visiting German-Nepal Tuberculosis Project (GENETUP) hospital. A total of 130 sputum specimens from were collected. All collected sputum specimens were processed for microscopy and some for culture and LAMP. During sample collection, patients were instructed to take a deep breath and coughed deeply and vigorously. Patients were instructed to cover their mouths carefully while coughing and not to use oral antiseptics during the period of sample collection. Saliva and nasal secretions were not accepted (Forbes et al., 2000). Similarly sputum containing food particles, residues and other extraneous matter were also rejected.

For the new patients suspected of pulmonary tuberculosis, three sputum samples were taken, one "on the spot" during the time when patients visited to the hospital, followed by a two sputum sample on the next days. First at the early morning and then third on the spot (Maher et al.,1997). Only one sample was taken from the follow-up patients. All the samples were collected in leak proof, wide mouth, and transparent, sterile and stopper plastic container.

3.2.3 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.

i) Digestion, Decontamination and Concentration

Digestion, Decontamination and Concentration were done as per standard methodology Briefly,

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

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

1) MycoPrep NaOH-NALC sodium citrate solution was added in a volume equal to the quantity of specimen. Tighten the cap.

2) Vertex lightly or hand mix for about 15-30 seconds. The tube was then inverted to expose the whole solution with NALC-NaOH solution.

3) Kept at room temperature, with gentle continuous shaking/rotation for 15 minute. After completion of shaking, it was stand at room temperature for 5 minutes to allow for settling of aerosols.

4) Specimen volume was made up to 50 ml with the addition of sterile MycoPrep phosphate buffer (Appendix III), ensuring that there is no cross-contamination. Mixed well.

5) The specimen was centrifuged at 3000 X g for 20 minute at 4-16°C.

6) After centrifugation, the tube was left for 5 minutes to allow aerosols to settle.

7) In the class II biosafety cabinet, the supernatant was carefulluy decant into a suitable container containing a mycobactericidal disinfectant.

8) Using a sterile pipette, pellet was resuspended with 1 ml sterile phosphate buffer and lightly vortex or mixed with the pipette.

9) The resuspended pellet was then used for smears, LJ culture and DNA extraction.

ii) Sputum Microscopy

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

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

Microscopic Examination of Smears by Flurochrome (FL) method.

1. The heat fixed slides were placed on a staining rack, with smears facing up.
2. The Rhodamine-auramine solution was poured on the slide to cover the entire smear and allowed to stand for 15 min.

3. After 15 minute the slides were washed with tap water and drained.

4. The slides were then decolorized 20% sulphuric acid and leave for 2 minutes.

5. The slides were gently washed with water again and drained.

6. The slides were covered with counter stain (e.g., Potassium permanganate) solution and left for 2 minutes.

7. The slides were gently washed with water and drained.

8. Then after, the slides were air- dried and the whole smear was examined under 10X and 40Xusing a UV light microscope for acid-fast bacilli.

The organisms appeared as yellowish rods against a dark background.

iii) Sputum Culture

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

For culture, using the pipette used for adding phosphate buffer to the pellet,2-3 drops (0.1-0.2 ml) of concentrated and decontaminated sputum was inoculated into each of two LJ slants. The slants were laid with medium face up for 30 minutes to allow the bacteria to adhere to the surface of the medium. The tubes were incubated at 37°C for up to 8 weeks. Examined for growth twice weekly for 4 weeks and then once weekly for up to 8 weeks. If any colonies were seen at any stage, acid fastness of bacilli was determined by smear examination of the growth. Contamination was recorded when it was apparent. Negative report was given when no colonies appeared after observing weekly for 8 weeks.

iii) Loop-Mediated Isothermal Amplification (LAMP)

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

a) DNA Extraction

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

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

Thus prepared DNA was used for PCR.

b) LAMP reaction

Number of cycle: LAMP was performed in a total 25 μl 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^oC for one hour.

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

Primers

F3: 5' CTGGCTCAGGACGAACG 3'

FIP: 5' CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT 3'

FL: 5' GTTCGCCACTCGAGTATCTCCG 3'

BL: 5' GAAACTGGGTCTAATACCGG 3'

BIP: 5' TCGGGATAAGCCTGGACCACAAGACATGCATCCCCT 3'

B3: 5' GCTCATCCCACACCGC 3'

LAMP was performed in a total 25 μl reaction mixture containing 10 x LAMP buffer, 14 mM dNTPs, 5 M betaine, 100 mM $MgSO_4$, primer mix (F3, B3, BIP, FIP, loop F, loop B), *BST* DNA polymerase, distilled water, DNA samples, and 1/10-diluted original

SYBR Green I for the direct detection of LAMP amplicons in the reaction tube with naked eye.

c) Observation of Results

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

3.4. Statistical analysis and Interpretation of the Results

The solution turned green in the presence of amplification product indicating positive result, while it remained orange with no amplification indicating negative result. Large amount of amplicons when bind with SYBER Green I, it fluoresces green indicating target gene amplification. SPSS soft was used to analyse the data.

CHAPTER IV: RESULT

A total of 130 sputum samples from two different study groups were collected in this study. All the suspected TB patient's sputum specimens were collected and examined by Microscopy. Sputum specimens were further examined by culture and LAMP. The results obtained by culture and microscopy were used for evaluating the specificity and sensitivity of the LAMP over the sputum smear microscopy and culture.

4.1 Laboratory Result

In this study, a total of 130 sputum samples were collected from two different study groups.

4.1.1 Microscopy

From this group, all sputum specimens were examined by fluorochrome staining. Out of 130 (100%) sputum specimens, 55 (42.63%) were positive and remaining 74 (57.36%) were negative by fluorochrome staining.

4.1.2 Culture

From 130 sputum specimens, a total of 69 (100%) sputum specimens were examined by culture according to culture facility at the laboratory. Among 69 sputum specimens, 40 (57.97%) were positive by culture while remaining 29 (42.03%) sputum specimens were negative by culture.

4.1.3 LAMP

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

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

Table 1: Comparative Results of 69 sputum specimens with microcopy, culture and LAMP

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
34 (49.27%)	35 (50.73%)	40 (57.97%)	29 (42.03%)	43 (62.32%)	1 (1.45%)	-	-	44 (63.77%)	25 (36.23%)

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

4.1 .4 Study group B

This group includes 61 sputum specimens from 61 follow up patients visiting National TB Reference Lab of GENETUP, kalimati. From this group all sputum specimens were examined by Microscopy, culture and LAMP.

4.1.5 Microscopy

All the sputum specimens from study group B were examined by flourochrome staining. Out of 61(100%) specimens, 20 (31.79%) were positive and 41 (67.21%) were negative by fluorochrome staining.

4.1.6 Culture

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

4.1.7 LAMP

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

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

Table 2: Comparative Results of 61 sputum specimens with microcopy, culture and LAMP

Microscopy		Culture		Loop-Mediated Isothermal Amplification (LAMP) result					
+ve	-ve	+ve	-ve	+ve					-ve
				MTB	MIN	MAV	MK	Total	
20 (32.78%)	41 (67.22%)	31 (50.82%)	30 (49.18%)	33 (54.1%)	1 (1.64%)	-	-	34 (55.74%)	27 (44.26%)

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

4.1.8 Comparative results of total sputum specimens

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

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

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

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

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

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

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

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

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

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

Table 6: Age and Sex wise distribution of total *M. intracellular* cases

Age Group (Years)	Male		Female		Total	
	Number	%	Number	%	Number	%
10-20	0	0	0	0	0	0
21-30	0	0	0	0	0	0
31-40	0	0	0	0	0	0
41-50	0	0	0	0	0	0
51-60	1	50	0	0	1	50
Above 60	1	50	0	0	1	50
Total	2	100.00	0	0	2	100.00

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

4.2 Quality Control

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

Table 7: Evaluation of Primers

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

For this purpose we used five tubes each containing LAMP reagent and positive control of *M. tuberculosis, M. avium, M. intracellular,M. kansasii* and tube without DNA template respectively for evaluation of one primer. LAMP was performed by using only

one primer at once and observed for species-specificity but no such cross-reaction was found.

4.3 Evaluation of Tests:

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

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

Table 8: Comparison of LAMP with reference to culture

<table>
<tr><td rowspan="4">L
A
M
P</td><td rowspan="2">Test and Results</td><td colspan="2">Culture</td><td rowspan="2">Sensitivity</td><td rowspan="2">Specificity</td><td rowspan="2">PV+</td><td rowspan="2">PV-</td><td rowspan="2">False - ve</td><td rowspan="2">False +ve</td></tr>
<tr><td>+ve</td><td>-ve</td></tr>
<tr><td>Positive</td><td>67</td><td>11</td><td rowspan="2">94.36%</td><td rowspan="2">81.36%</td><td rowspan="2">85.90%</td><td rowspan="2">92.31%</td><td rowspan="2">5.63%</td><td rowspan="2">18.64%</td></tr>
<tr><td>Negative</td><td>4</td><td>48</td></tr>
</table>

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

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

Table 9: Comparison of LAMP with reference to microscopy

<table>
<tr><td rowspan="4">L
A
M
P</td><td rowspan="2">Test and Results</td><td colspan="2">Microscopy</td><td rowspan="2">Sensitivity</td><td rowspan="2">Specificity</td><td rowspan="2">PV+</td><td rowspan="2">PV-</td><td rowspan="2">False - ve</td><td rowspan="2">False +ve</td></tr>
<tr><td>+ve</td><td>-ve</td></tr>
<tr><td>Positive</td><td>53</td><td>25</td><td rowspan="2">98.14%</td><td rowspan="2">67.11%</td><td rowspan="2">67.94%</td><td rowspan="2">98.07%</td><td rowspan="2">1.85%</td><td rowspan="2">32.89%</td></tr>
<tr><td>Negative</td><td>1</td><td>51</td></tr>
</table>

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

CHAPTER V: DISCUSSION AND CONCLUSION

The rapid detection and identification of mycobacterial pathogens in sputum samples are necessary for the effective treatment of tuberculosis as well as pulmonary disease due to atypical mycobacteria. For this purpose we used a novel nucleic acid amplification method, loop-mediated isothermal amplification method for detection of *M. tuberculosis* complex, *M. avium. M. intracellular* and *M. kansasii* in the sputum samples. The samples were subjected to fluorochrome staining for detection of the acid-fast bacilli followed by culture on Lowenstein- Jensen medium and LAMP for the detecting *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii*. Then the sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive were calculated to compare LAMP with cultural and microscopic methods of diagnosis. This is the first research in our country in which LAMP has been used for the diagnosis of *M. tuberculosis, M. avium, M. intracellular* and *M. kansasii.*

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

The Loop-Mediated Isothermal Amplification (LAMP) operation is quite simple. It starts with the mixing of the buffer primers, DNA lysates and DNA polymerase in tube, and then the mixture is incubated at 64°c for 1 hour. There is no necessity for heat denaturation of the template DNA. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of

64ºc. Visual judgment eliminates the need for any laboratories and time consuming post amplification operations such as hybridization and electrophoresis as well as the need for special equipment.

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

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

During the study period, a total of 130 sputum specimens were included to compare LAMP result with gold standard culture and Microscopy. Among the studied 130 sputum samples, 54 were fluorochrome staining positive. Out of 54 smear positive sampes, 51 showed growth on Lowenstein-jensen medium but the remaining 3 showed no growth on this medium which were positive by LAMP. This may be due to the over decontamination of sputum sample which may kill the organism and thus these samples showed no growth on the culture medium. Similarly 76 sputum samples were fluorochrome staining negative. Out of them, 20 samples were positive while culture on Lowenstein-Jensen medium. This may be due to lower number of organism present in the sputum sample. Less than 5,000-10,000 organisms per ml are not detected through the microscopy but even 100 organisms per ml can be detected by the solid medium. .

By culture, 71 sputum samples showed growth on the Lowenstein-Jensen medium. Upon testing by LAMP, 67 culture positive samples were positive indicating high sensitivity of

LAMP. Remaining 4 culture positive samples were negative by LAMP, this may be due to inefficient lysis of cells during freezing and thawing process for DNA extraction. The negative result may also be due to using primers targeting few species only. In this study we used primers targeting only four Mycbactrial species (*M. tuberculosis*. *M .avium*,. *M. intracellular* amd *M. kansasii)*. The mycobacteria grown on the culture media may be *M. xenopi*, *M.fortuitum* and other atypical mycobacteria which could not be detected by LAMP in this study.

Among 59 culture negative sputum samples 11 were positive by LAMP. From these 11 cultre negative samples, 8 were negative by smear also. Most of these samples were from follow up patients who were under treatment. The possible cause of culture negative but LAMP positive result may be due to fast sputum conversion rate by culture than PCR among follow up patients due to effect of anti TB drug. Another possible cause for that type of result may be due to over decontamination of sputum specimens.

In this study, LAMP was performed by using primers targeting *M. tuberculosis*, *M. intracellular*, *M. avium* and *M. kansasii*. Among 78 LAMP positive samples, 76 were positive with *M. tuberculosis* primer and remaining 2 were positive with *M. intracellular* primer. None of the *M. avium* and *M. kansasii* cases were found during the overall study period. Tuberculosis infection was distributed among all age group with highest in 21-30 years but *M. intracellular* -pulmonary disease was found among elderly people. Among them one was found in new cases and another was found from was found in follow up patient. This might be due to weak immunologic function of old age people and people with pre-existing lung disease.

While assessing the accuracy of the diagnostic test, sensitivity and specificity are very important components to be considered. In this study, LAMP has been compared with culture and microscopy taking them as reference methods. Further the accuracy of the test has also been evaluated by calculating predictive value of positive test, predictive value of negative test, Percentage of false negative and percentage of false positive. While comparing the LAMP with culture as gold standard, the sensitivity of LAMP was 94.36 %, specificity was 81.36 %, predictive value of positive value was 85.90 %, predictive value of negative value was 92.31 %, percentage of false positive was 5.63% and percentage of false positive was 18.64 % respectively. This demonstrates the high

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

The possibilities of finding low specificity as compared with culture are supported by some other literatures. Vladimirskiy et al. (2003) used the PCR assay of MTB detection for to observe efficiency of specific chemotherapy of the patients with pulmonary tuberculosis. 18 from 21 observed patients analyzed for MTB excretion in sputum during 4-7 months every other 1.5 months by the culture on Lowenstein-Jensen solid medium and PCR assay exhibited a positive clinico-radiological changes and sputum conversion at the end of term of observation. In 4 patients with small processes without lung destruction MTB was not defined sputum by culture but it was found at 3 patients by PCR. These patients became to negative in 3 months of treatments. From 11 patients with infiltrative form with lung destruction MTB was finding at 9 cases by culture method in comparison with 11 patients in whom MTB was detected by PCR. The sputum conversion by culture these patients was occurred on the average in 1.8 months, in comparison with 4.7 months by PCR. 3 patients with fibro-cavernous tuberculosis of lungs have exhibited a sputum conversion at 3 months by culture method and 5.7 months at average by PCR.

With reference to Microscopy, the sensitivity of LAMP was 98.14%, indicating high accuracy rate of LAMP and specificity was 67.11%. The lower specificity of LAMP as compare with Microscopy may be due detection limit of organisms by Microscopy. Lower number of organisms (Less than 5,000-10,000) in the sputum can not be detected by smear microscopy which may be positive by LAMP.

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

Enosawa et al. (2003) evaluated the usefulness of LAMP in detecting specific gene sequences of cultured *M. avium* subsp. *Paratuberculosis* (MAP). A total of 102 primer sets for LAMP was designed to amplify the IS900,HspX and F57 gene sequences of MAP. Using each of two primer sets (P-1 and P-2) derived from the IS900fragment; it was possible to detect MAP in a manner similar to that used with nested PCR. The sensitivity of LAMP with P-1 was 0.5pg/tube, which was more sensitive than nested PCR. When P-2 was used, 5pg/tuve could be detected, which was the same level of sensitivity as that for nested PCR. LAMP with P-1 was specific. Although only 2 *M. scrofulaceum* strains out of 43 non- MAP mycobacterial strains were amplified, the amplification reaction for these strains was less efficient than for MAP strains, and their products could be distinguished from MAP products by restriction digestion. LAMP with P-2 resulted in very specific amplification only from MAP, the same result obtained with nested PCR. These results indicate that LAMP can provide a rapid yet simple test for the detection of MAP.

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

compared with investigators who applied LAMP for diagnosis of diseases other than tuberculosis

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

Kuboki et al. (2003) reported conditions for a highly sensitive, specific and easy diagnostic assay based on LAMP technology for the detection of parasites in the *Trypanosoma brucei* group (including *T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, and *T. evansi*) and *T. congolense*. They showed that he sensitivity of the LAMP based method for detection to trypanosomes in vitro was up to 100 times higher than that of PCR based methods.

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

Identification of the species of mycobacterial isolates is another critical requirement for clinical laboratories because most first line antituberculosis drugs have less invitro activity against *M. avium* complex and *M. kansasii*. Identification of Mycobacteria in most of the hospitals and research laboratories in our country do not reach up to the species level. All suspected tuberculosis cases, when showed growth on culture media, referred to as tuberculosis and physician start treatment of tuberculosis. These activities are the main burden for the effective control and management of tuberculosis. The conventional biochemical tests for identification of mycobacteial species are time-consuming because of the slow growth of mycobacteia on culture media. The LAMP-

based assay can identify *M. tuberculosis* complex, *M. avium*, *M. intracellular* and *M. kansasii* from sputum samples directly within 1 hour. Hence the LAMP assay is more advantageous than all of the currently available DNA probe methods in its simple operation and rapid reaction.

CHAPTER VI: CONCLUSION AND RECOMMENDATION

Conclusion

In conclusion, the LAMP-based assay used in this study is novel nucleic acid amplification method that allows rapid and accurate identification of *M. tuberculosis*, *M. avium*, *M. intracellular* and *M. kansasii* in sputum samples. Due to its easy operation without sophisticated equipment, it will be simple enough to use in small-scale hospitals, primary care facilities and clinical laboratories in developing countries if problems such as during sample preparation, nucleic acid extraction and cross contaminations are addressed.

Recommendation

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

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

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

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

CHAPTER VII: REFERENCES

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CHAPTER-VIII: ANNEXES

Annex I

A) Bacteriological media

Lowenstein-jensen Medium (L-J Medium)

B) Reagents/ Chemicals

Absolute ethanol

Auramine-O

Acid-alcohol

Buffer

Bst DNA Polymerase

Betaine

dNTPs(dATP, dTTP, dCTP, dGTP)

Distilled water

Glycerol

Lysol

Magnesium Sulphate

Malachite green

Methylene Blue

N-acetyl-L-cysteine

primer

Sodium citrate

Sodium hydroxide

C) Reagents and solution for LAMP

LAMP Buffer

$MgSO_4$

Betaine (N,N,N-trimethylglycine)

Deoxyribonucleoside triphosphate (dNTPs)

Bst DNA polymerase.

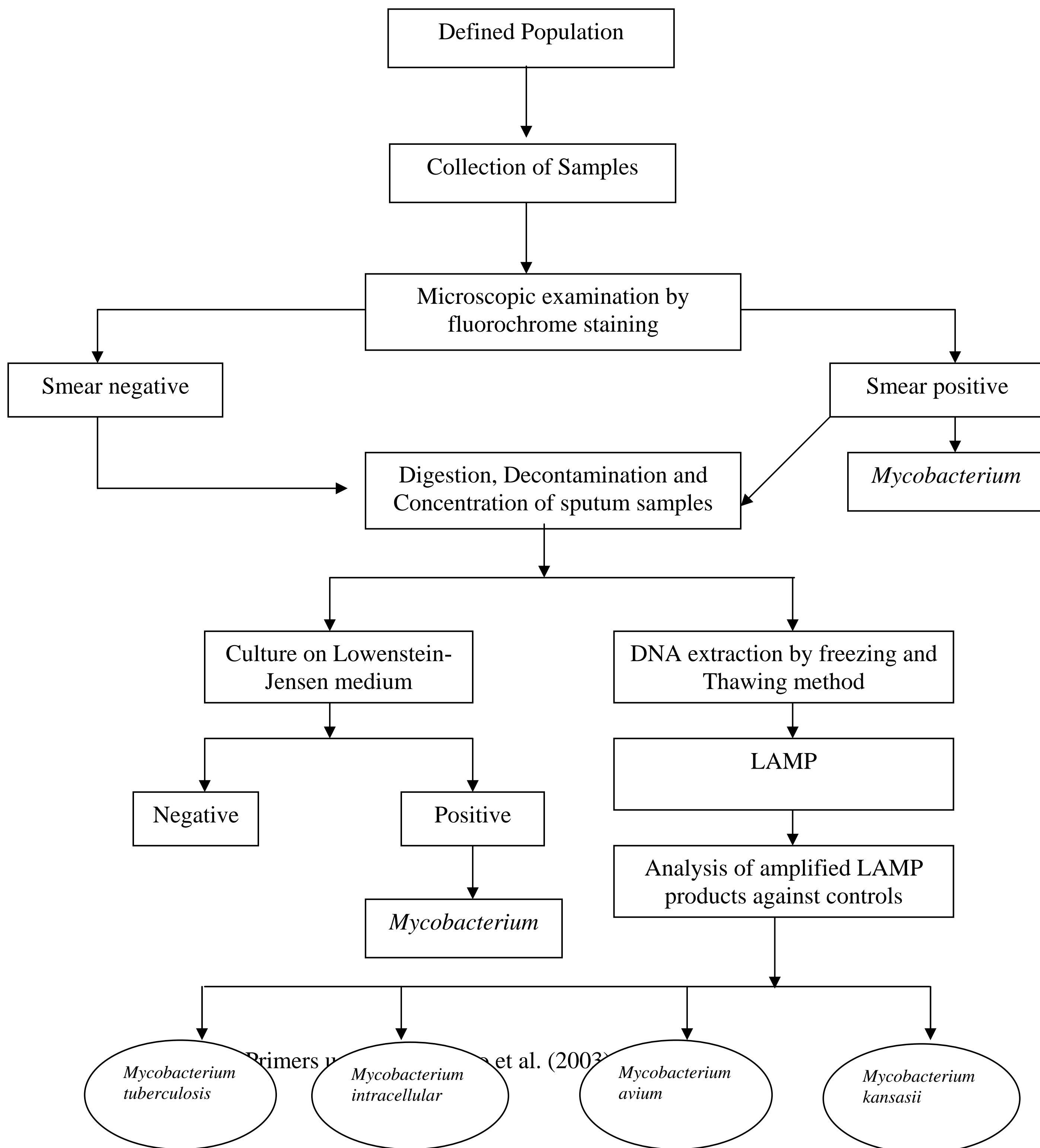
Primer

Template DNA

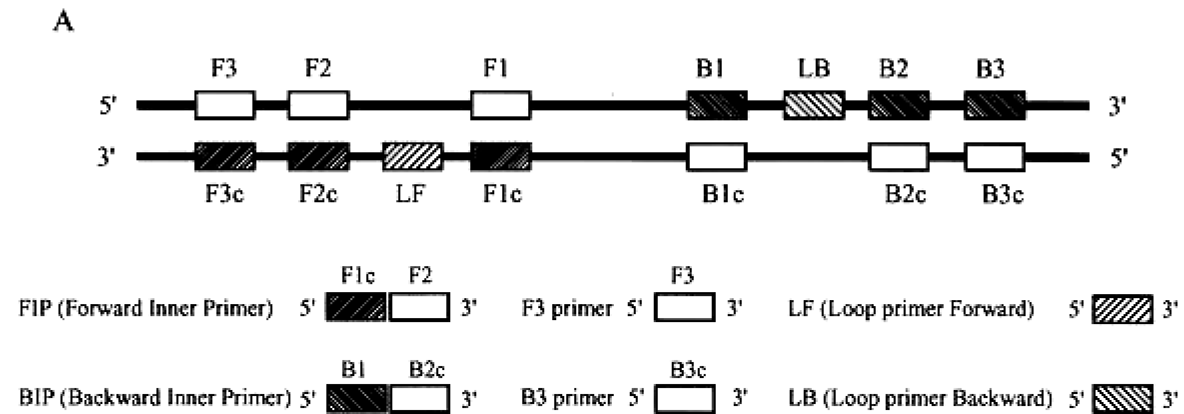
SYBR Green I

4. Primers (Invitrogen T_m life technologies)

Annex II: Flow Chart of Methodology



Annex III: Primer used



B

MTB primers:

Gcgatatctggtggtctgca cggcgtcggc gtgtCggtgg ttaacgcgct aTccacccGg ctcgaagtcg
F3 F2 loop F

agatcaAgcG cgacgggtac gagtggtcTc aggtttAtga gaagtcggaa cccctggGCc tcaagcaagg
F1c B1 loop B

ggcGccgacc aaGaagacgg ggtcaacggT gcggttctgg gccgaccccG ctgttttcga aaccacgG
B2c B3c

MAV primers:

Gcctgaccat caacctcaCc gacgagCggg tgaccaacga agaGgtcgtc gaCgaggtgg tcagcgacAc
F3 F2 loop F

cgccgacgca cCcaagtcgg cgcaggagaa GgcggcggAa tcggctgcgc cgcataaGgT caagcaccgc
F1c B1 loop B

accttCcact accccggcgg Cctggtcgac ttcgtcaaAc acatcaatcg caCcaaaaac cccatccacc A
B2c B3c

MIN primers:

Acgaggtcgt cagcgataCc gccgacgcac cCaagtcggc ccaggaaaAg Gcggcggaat cgactGcGcc
F3 F2 loop F

acataaggtt aagcaccgCa ccttccacta ccccggcggt ctggtcgact Tcgtcaagca catcaaccgcA
F1c B1

ccaaGagcc cgatccagca gAgcatcatc Gacttcgacg gcaaaggtCc cggccacgag gtcgagatcg
loop B B2c

cgatgcagtg gaacggcgGc tactcggaat ccgtgC
B3c

Muniv primers:

Gcggtgtggg atggGcCcgc ggcctatcag Cttgttggtg gggtgacGgc ctaccaaggc gaCgaCgggt
F3 F2 loopF

agccggcctg agAgggtgtc cggccAcact gggactgaga tacggcCcAg actcctacgg gaggcaGcag
F1c B1 loop B

tggggaatat tgcacaatgg Gcgcaagcct gatgcAgcga cgccgcgtgg ggGatgacgg ccttcggG
B2c B3c