Isolation of *Mycobacterium leprae* DNA from Slit Skin Scrapings of Nepalese leprosy patients and determination of Variable Number of Tandem Repeat Polymorphism by DNA Fragment Length Analysis.

A

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BY

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RECOMMENDATION

This is to certify that **Mr. Jivan Shakya** has completed this dissertation work entitled **"Isolation of *Mycobacterium leprae* DNA from Slit Skin Scrapings of Nepalese leprosy patients and determination of Variable Number of Tandem Repeat Polymorphism by DNA Fragment Length Analysis"** as a partial fulfilment of M.Sc. degree in Microbiology under our supervision. To our knowledge this work has not been submitted for any other degree.

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ABSTRACT

Nepal is a leprosy endemic country. Global standard methods to decipher *Mycobacterium leprae* strain information have been based on VNTR polymorphisms detected by DNA sequencing and DNA fragment length analysis (FLA). Herein, DNA fragment length analysis was employed to detect polymorphisms at 18 different VNTR loci within Nepalese *M. leprae* isolates. 40 slit skin scraping (SSS) samples from MB patients used routinely for diagnosis of leprosy were collected for *M. leprae* DNA isolation. Each DNA sample was then amplified by multiplex-PCR in four combinations containing different primer pairs of 18 different VNTR loci. The VNTR loci amplified in this study were (AC)8b, (GTA)9, (GGT)5, (AT)17, rpoT (6-3a), 21-3, (AC)9, (AT)15, (AC)8a, ML01, 27-5, 6-7, (TA)18, (GAA)21, 18-8, 12-5, 23-3 and (TA)10. The lengths of the amplicons were then analyzed by DNA fragment analysis to determine the correct number of repeat units at each of the VNTR loci. The consolidated data on each *M. leprae* isolate was then used to classify 40 *M. leprae* isolates into various clusters of similarity. DNA was successfully isolated from SSS samples. The $A_{260/280}$ ratio ranged from 0.83 to 1.45 and the yield ranged from 10.1 μg/ml to 265.5 μg/ml. Preliminary data on VNTR polymorphisms from 40 samples indicated that locus *rpoT* was considerably stable in Nepal with repeat copy number 3. (TA)18 and (AT)15 were the most polymorphic of all and showed 12 and 11 different alleles correspondingly. In general, minisatellite loci were found to be more stable than microsatellite loci. Based on the similarity of VNTR patterns, the 40 *M. leprae* isolates could be separated into four major clusters and four unique strains. In one instance, the samples from the same family showed similar patterns at the VNTR loci. These results indicate that these methods can be used for strain typing of Nepalese *M. leprae* isolates according to globally recognized standards.

Key Words : Leprosy, *M. leprae*, Variable Number of Tandem Repeats (VNTR), Multiplex-PCR, DNA Fragment Length Analysis, Slit Skin Scraping, VNTR polymorphisms, Strain typing.

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
ATP	Adenosine Triphosphate
BB	Mid-borderline
BI	Bacillary Index
BL	Borderline Lepromatous
bp	base pairs
BT	Borderline Tuberculoid
CDR	Central Development Region
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
EDR	Eastern Development Region
EDTA	Ethylene Diamine Tetra Acetate
ELISA	Enzyme Linked Immunosorbent Assay
ENL	Erythema Nodosum Leprosum
FLA	Fragment Length Analysis
FWDR	Far Western Development Region
IFN-γ	Interferon-gamma
IL	Interleukin
IS	Insertion Sequence
kDa	kilo Dalton
LL	Lepromatous Leprosy
MB	Multibacillary
MDT	Multi drug therapy
MHC	Major Histocompatibility Complex
MLVA	Multiple Locus VNTR Analysis
NADH	Nicotinamide adenine dinucleotide hydrogen
NHDP	National Hansen's Disease Program
PAGE	Polyacrylamide Gel Electrophoresis
PB	Paucibacillary
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

PGL-I	Phenolic Glycolipid I
PR	Prevalence Rate
RFLP	Restriction Fragment Length Polymorphism
RR	Reversal Reaction
rRNA	ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeats
STR	Short Tandem Repeat
T1R	Type 1 reaction
TBE	Tris Borate EDTA
TEMED	Tetra Ethane Methylene Diamine
TLR	Toll like receptors
TN	Tamil Nadu
TNF- α	Tumor Necrosis factor – alpha
TR	Tandem repeats
TT	Tuberculoid leprosy
UV	Ultra Violet
VNTR	Variable Number of Tandem Repeats
WDR	Western Development Region
WHO	World Health Organization
µl	microlitre
µg	Microgram
ml	Millilitre

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CHAPTER 1

Introduction

Based upon clinical descriptions, leprosy has afflicted man and from ancient times was considered a communicable disease (Hastings, 1985). Leprosy or Hansen's disease is a chronic infectious disease caused by *Mycobacterium leprae,* an obligate intracellular pathogen (Hastings *et al.*, 1988; Scollard *et al.*, 2006). Despite being one of the oldest diseases, leprosy is still prevalent in the world as indicated by 213,036 registered leprosy patients at the beginning of 2009 and 249007 new cases identified globally during 2008. Since the implementation of multi-drug therapy (MDT) in 1982 by World Health Organization (WHO) to combat against leprosy, the prevalence of the disease has considerably decreased. However, the decrease in new case detection rate has been less steep (WHO, 2009a).

The mode of leprosy transmission has remained poorly understood. Multiple modes of transmission have been proposed, but no single method has yet been definitively proven to transmit the disease. Nasal secretion in the form of droplets has been considered significant since *M. leprae* has been detected from nasal mucosa of multi-bacillary patients (Klatser *et al.*, 1993; Rees and McDougall, 1977; Smith *et al.*, 2004). Similarly direct skin contact between humans (Fine *et al.*, 1997; Noordeen, 1994), vector borne (Meyers *et al.*, 1992; Walsh *et al.*, 1988) and vehicle borne (Matsuoka *et al.*, 1999) have been considered possible mechanisms. Interestingly, a free living amoeba *Acanthamoeba castellanii,* has also been implicated in leprosy transmission (Grange *et al.*, 1987; Lahiri and Krahenbuhl, 2008). In contrast to its fastidious requirement of intracellular niche for multiplication, *M. leprae* has been shown to remain viable in the environment outside of a human host (Desikan and Sreevatsa, 1995).

In particular, the pattern of transmission within local communities and global populations remains yet to be elucidated (Matsuoka, 2009). Strain typing has potential applications in such situations to trace the geographical distribution and epidemiological relatedness of etiological agents (Riley, 2004). Decoding of genome sequence of *M. leprae* TN strain in 2001 (Cole *et al.*, 2001a) has subsequently led to the identification of

multiple polymorphic loci with potential for strain typing (Groathouse *et al.*, 2004; Scollard *et al.*, 2006; Young *et al.*, 2004; Zhang *et al.*, 2005).

Global initiatives for typing of *M. leprae* strains have been based on polymerase chain reaction (PCR) amplification of a panel of identified variable number of tandem repeats (VNTR) loci, a method known as Multiple Loci VNTR Analysis (MLVA). A 6-bp intragenic sequence in *rpoT* gene and a trinucleotide TTC repeat element upstream of a pseudogene were the first ones to be shown polymorphic or exhibiting a variable number of tandem repeats when sequenced in different isolates (Matsuoka *et al.*, 2000; Shin *et al.*, 2000). Application of VNTR polymorphism as a tool for strain typing has been well recognized for other bacterial strains like *Legionella pneumophila* (Nederbragt *et al.*, 2008), *Bacillus anthracis* (Keim *et al.*, 2000), *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli O157, Salmonella enterica* (Malorny *et al.*, 2008), *Yersinia pestis* (Klevytska *et al.*, 2001), *Mycobacterium tuberculosis* (Frothingham and Meeker-O'Connell, 1998; Spurgiesz *et al.*, 2003; Supply *et al.*, 2000) and *Mycobacterium bovis* (Skuce *et al.*, 2002) .

Initially, the typing scheme for *M. leprae* was based upon polymorphisms detected at a single locus. The resulting data demonstrated a wide range of allelic diversity, as with the case of TTC loci, and hence was considered insufficient to discern relatedness among strains owing to low discriminatory power based on single loci (Truman *et al.*, 2004). Identification of new multiple polymorphic VNTR loci (Groathouse *et al.*, 2004; Zhang *et al.*, 2005) has led other workers to search for even more informative loci for typing *M. leprae* strains (Gillis *et al.*, 2009; Kimura *et al.*, 2009; Young *et al.*, 2004; Young *et al.*, 2008; Zhang *et al.*, 2005).

Nepal is an endemic country for leprosy and lacks robust information on strains circulating within the country. Previous attempts by Thapa and Adhikari sought to quantitate the allelic diversity of *M. leprae* strains from Nepal and the probable relatedness among the strains. Their method was based upon PCR employing 6 minisatellite and 6 microsatellite loci within the *M. leprae* genome, Polyacrylamide gel electrophoresis (PAGE) analysis with evaluation by graphic based computer software.

The analysis was later determined to be too crude to accurately identify the number of VNTR alleles at a locus. Use of DNA sequencing technology is currently considered the gold standard to correctly reveal number of repeats at VNTR loci. But in the absence of which, DNA Fragment length analysis (FLA) technique could have the same advantage in reporting the predominant alleles (Kimura *et al.*, 2009).

This study aims to test the implementation of the global starndard methods to characterize Nepalese *M. leprae* isolates based on VNTR polymorphisms. Similar studies conducted elsewhere in the world are based on the standard FLA technique to correctly estimate the number of repeats at the VNTR loci. This study seeks the establishment of the same technique in Nepal so that the data generated would be valid to be compared with the global data and further analysis. Previous similar studies conducted in Nepal had taken biopsy as sample of choice and single PCR reaction for each VNTR loci. The data on VNTR polymorphisms were then generated by an analysis of PAGE done by a graphic based web program. Subsequent analysis of the same by FLA technique showed incongruent results between the two. Hence, the previous method was thought to be too crude for such studies. This study will aim to utilize Slit Skin Scrapings (SSS) as a sample for *M. leprae* DNA isolation. Taking a SSS is easier compared to taking a biopsy. A multiplex PCR will then be performed for 18 different VNTR loci in four combinations for each isolate. So generated amplicons will then be analyzed by FLA technique to determine repeat number polymorphisms at each of the loci. This study aims to establish FLA technique as a method for carrying out VNTR based strain typing of *M. leprae* in Nepal.

Chapter 2
Objectives

General Objectives:

1. To identify *Mycobacterium leprae* strains from Nepali leprosy patient SSS using VNTR strain typing by multiplex PCR followed by DNA fragment length analysis.

Specific Objectives:

1. Isolate *M. leprae* DNA from Nepali leprosy patient SSS.
2. Amplify VNTR at 18 different genomic loci using multiplex PCR.
3. Analyze the length of amplicons and repeat numbers by DNA fragment analysis to determine variations at 18 VNTR loci of *M. leprae* genome
4. Compare resulting patient and molecular epidemiological results with similar applications within leprosy endemic populations.

Chapter 3
Literature Review

3.1 Leprosy

Leprosy is a disease involving chronic infection of the skin and the peripheral nerves by an intracellular bacteria *Mycobacterium leprae* (Britton and Lockwood, 2004). The disease also affects mucosa of the upper respiratory tract and the eyes (WHO, 2010a). Leprosy is curable and early treatment checks the progress towards disability (WHO, 2010a).

3.2 Epidemiology of Leprosy

Leprosy seems to have been present among human populations since ancient times as evidenced by the finding of the cases in China, India and Egypt around 600 BC and the skeletal remains bearing hallmarks of the disease found in Egypt (Hastings, 1985) More recent explanations on global dissemination of leprosy have been provided by Monot *et al*. (2005) based on Single Nucleotide Polymorphisms (SNP) within the *M. leprae* genome. SNP type 2 strains are thought to have existed within East Africa and Central Asia, preceding SNP type 1 and SNP type 3. The SNP type 1 migrated towards east and the SNP type 3 migrated westwards to North Africa and Europe from where the disease spread to the Americas. Towards West Africa, the disease is believed to be introduced by infected explorers, traders and colonialist from European or North African descent rather than emigration from East Africa because the prevalent SNP type 4 is more closely related to SNP type 3 found in North African and European regions than SNP type 2 found among East African regions. The disease was then introduced to Brazil and Carribean islands as a direct consequence of slave trade during 18^{th} century as evidenced by the finding of the same SNP type 4 in both regions.

After decades of interventions, leprosy still prevails in the world as a major health problem. Pockets of endemicity still exist around the world as in some areas of Angola, Brazil, the Democratic Republic of the Congo, India, Madagascar, Mozambique, Nepal and the United Republic of Tanzania (WHO, 2010a). At the beginning of 2009, the global prevalence of leprosy was 213,036. The registered prevalence is calculated

according to the number of patients actively receiving multi-drug therapy (MDT). During the year 2008, the number of new cases detected as reported by 121 countries was 249,007 (WHO, 2009b). Similarly, at the end of first quater of 2010 (Table 1), the registered prevalence of leprosy was 211903 cases as reported by 141 countries (WHO, 2010b) and 244796 new cases were detected during 2009 globally (WHO, 2010b). Comparison with the data of previous years has shown decline in new case detection rate from 620,638 cases during 2002 to 244796 cases during 2009 (WHO, 2009b, 2010b). However, the decreasing trend in new case detection does not necessarily reflect reduced transmission of the disease (Smith and Richardus, 2008).

Table 1: Global leprosy situation (at the end of first quarter of 2010). (WHO, 2010b)

WHO Region	**Registered prevalence (beginning 2010)**	**New Cases detected (during 2009)**
African	30,947	28,935
Americas	43,370	40,474
South-East Asia	120,456	166,114
Eastern Mediterranean	8,495	4,029
Western Pacific	8,635	5,243
Total	**211,903**	**244,796**

Nepal was one of the countries reporting more than 1000 new cases during 2008 and accounted for 1.89% of the total new cases detected during the year (WHO, 2009b). During 2009, there were still 4394 new cases of leprosy detected in Nepal (WHO, 2010b). All together 16 countries were reporting more than 1000 new cases of leprosy during 2009 including India, Brazil, Bangladesh, China, Sri Lanka, Democratic Republic of Congo and Indonesia (WHO, 2010b).

South-east Asia still hosts the bulk of total leprosy cases detected (Table 2). 166,114 new cases of leprosy were detected during 2009 in South-East Asia (Table 1) and 4394 new cases in Nepal during the same year (Table 2). However, the prevalence at the end of the year depicts a smaller number of patients. This discrepancy in the data is because

the prevalence is calculated at the end of the year by counting the number of leprosy patients actively taking MDT. This would obviously miss the paucibacillary (PB) patients detected early in the year who take MDT for 6 months and hence would not show up in the register as patients taking active MDT at the end of the year.

Table 2: Leprosy situation in South-East Asia (at end of first quarter of 2010). (WHO, 2010b)

Country	**Registered Prevalence (at end of first quarter of 2010)**	**New Cases detected (during 2009)**
Bangladesh	4163	5,239
Bhutan	30	12
India	87,190	133,717
Indonesia	21,026	17,260
Maldives	7	11
Myanmar	2,816	3,147
Nepal	2,445	4,394
Srilanka	1,849	1,875
Thailand	762	300
Timor-Leste	168	160
Total	**120,456**	**166,115**

Epidemiological interventions involving early case detection and case management with effective chemotherapy have formed the basis for global leprosy elimination strategies. Elimination in leprosy is defined as a prevalence rate (PR) of less than 1 case per 10,000 inhabitants. In the global context, the elimination target had been achieved by the end of 2000, the stipulated time set by World Health Assembly in 1991. However, Nepal only achieved the technical elimination prevalence level in November 2009 with prevalence rate of 0.89 per 10,000 populations (Fig. 1) (MoHP, 2009). 59 districts in Nepal have achieved the target PR of less than one while 16 districts have a PR between 1 and 3.

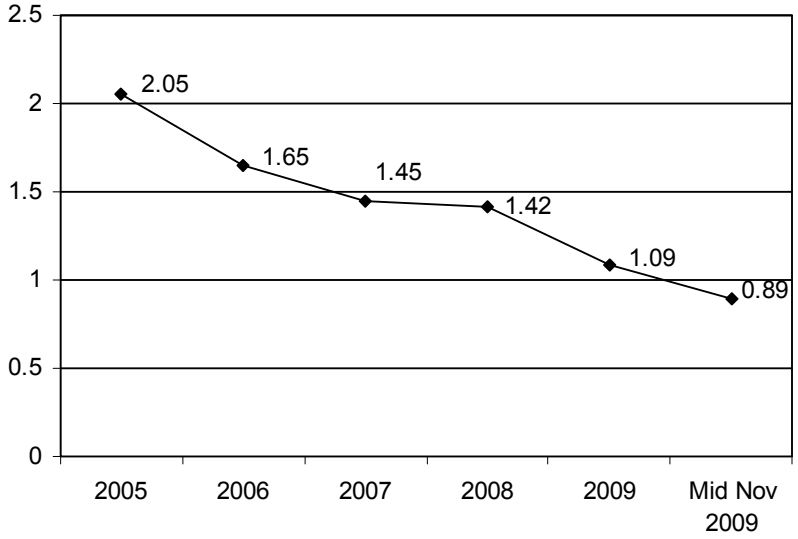


Fig 1: Prevalence Rate Trends of Leprosy in Nepal. Prevalence rate (calculated per population of 10,000) of leprosy from 2005 to mid-Nov 2009. (MoHP, 2009)

In Nepal, highest number of leprosy patients are distributed in the Central region (PR = 0.96) followed by the Eastern region (PR = 0.95) (Table 3) (MoHP, 2009). Most leprosy cases are concentrated in the Terai belt followed by hilly region (Table 4).

Table 3: Region-wise cases under treatment in Mid-November 2009, Nepal. Number of active cases of multibacillary (MB) and paucibacillary (PB) leprosy in different regions of Nepal as of mid-November 2009. (MoHP, 2009)

Region	No. of patients under treatment in Mid-November 2009		
	MB	PB	Total
EDR	425	176	601
CDR	551	368	919
WDR	296	79	375
MWDR	256	43	299
FWDR	193	58	251
National	**1721**	**724**	**2445**

Table 4: Eco-region wise distribution of leprosy in Nepal. Distribution of leprosy cases in Nepal according to geographical classification. (MoHP, 2009)

Type of district	Population	Percent of total population	Patients under treatment	Percent of total cases of the country
Terai	13432032	48.9%	1960	80%
Hilly	12097293	44.0%	424	17.50%
Mountain	1969260	7.1%	61	2.50%
Total	27498585	100%	2445	100%

3.3 Clinical Features

The clinical features of leprosy are dependent upon individual and varied host response to *M. leprae* (Walker and Lockwood, 2007). The variation in clinical manifestations is currently considered not due to different strains of *M. leprae* but is the result of the variation in the tissue response of the host to the presence of the leprosy bacilli in the body (Modlin, 1994). Basic cardinal features for diagnosis of leprosy include one or more of following :

- Hypopigmented anaesthetic skin patches or lesions
- Thickened peripheral nerves
- Slit skin smear positive for Acid Fast Bacilli (AFB)

Infection of peripheral nerves by *M. leprae* and the inflammatory and immunological response to the pathogen may subsequently lead to neuropathy in leprosy (Scollard *et al.*, 2006). Neuropathy then leads to the development of anesthesia, paralysis, and potential crippling deformities of fingers and toes or ocular damage in the case of facial nerve involvement (Scollard *et al.*, 2006).

3.4 Classification

Classification is important in leprosy in order to determine the course of treatment to be administered and predict the risk of complications among the patients (Walker and Lockwood, 2006). There are two systems to classify leprosy patients: clinical (WHO) and histopathological (Ridley-Jopling). Immunologically and histopathologically, the

disease has been classified into 5 groups over a spectrum (Ridley and Jopling, 1966) : two polar forms and three inbetween or borderline forms. Infection with *M. leprae* may either lead to self-healing or to an inderterminate form (I) which usually presents as a small hypopigmented erythematous macule with diminished sensation. Tuberculoid (TT) and lepromatous leprosy (LL) represent two polar forms of the disease with borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL) in between. TT patients will usually have one or two macular hypopigmented or erythematous anaesthetic lesions which have a well defined, often raised margin or occasionally are scaly plaques. LL disease is usually widespread at diagnosis and may consist of erythematous macules, papules and/or nodules or occasionally diffuse disease without distinct lesions. The lesions in borderline disease may be tuberculoid-like, but more numerous in BT disease, becoming more lepromatous in character in BL (Jacobson and Krahenbuhl, 1999). At the tuberculoid end, cell mediated immunity prevails and epitheloid granulomas with scarce or no bacilli predominate. At the lepromatous pole, lepromatous granulomas containing modified macrophages called lepra cells or virhowcytes are seen (Abulafia and Vignale, 1999).

The operational classification devised by WHO classifies leprosy into 2 groups : Paucibacillary (PB) and Multibacillary (MB), based on number of skin lesions (WHO, 1994). PB patients present with 1-5 skin lesions while MB patients commonly demonstrate 6 or more lesions. The I, TT and BT diseases are generally equivalent to paucibacillary disease while BB, BL and LL are generally equivalent to multibacillary disease (Jacobson and Krahenbuhl, 1999). However, in the field, BT cases are often clinically classified as either PB or MB depending on the individual patient's bacillary index (BI) and/or lesion counts.

3.5 Immunology of leprosy

Host defense mechanisms ensuing an early infection with *M. leprae* are poorly understood aspects of the immunology of leprosy (Scollard *et al.*, 2006). When a host mounts an effective innate immune response towards leprosy bacillus with low virulence, infection is checked and prevents the development of clinical disease (Scollard, 2006). In leprosy, clinical outcome is a direct consequence of host

immunological responses towards infection with *M. leprae* (Walker and Lockwood, 2006). Humoral immunity seems to be insignificant in limiting the proliferation of obligate intracellular pathogen *M. leprae*. Cellular immune reactions resulting in macrophage activation are considered more effective to protect the host (Hastings, 1985).

3.5.1 Innate Immunity

Dendritic cells (DC) are probably the first cell to encounter *M. leprae* at the site of invasion (Scollard *et al.,* 2006). DCs effectively uptake the mycoabacteria and present the antigenic information and have enhanced ability to poduce pro-inflammatory cytokines and chemokines (Demangel and Britton, 2000). Subsequent production of cytokines and chemokines then regulate inflammation and direct cell mediated immunity into T_H1 or T_H2 responses (Maeda *et al.*, 2003; Mittal *et al.*, 1989; Santos *et al.*, 2001). Studies have suggested the possible suppressive effect of infection with whole bacilli in the expression of MHC Class I and Class II molecules on monocyte-derived DCs, thereby limiting the interaction of DC and T cells (Hashimoto *et al.*, 2002).

Pattern recognition receptors further accentuate the innate immune responses by recognizing pathogen-associated molecular patterns (Gordon, 2004). C-type lectin receptors (Allavena *et al.*, 2004; Koppel *et al.*, 2004; van Kooyk and Geijtenbeek, 2003), Toll-like receptors (Bochud *et al.*, 2003; Kang and Chae, 2001) and Complement receptors (Schlesinger and Horwitz, 1991) have been shown to participate in innate immune responses to *M. leprae*.

3.5.2 Adaptive Immunity

Prolific local footpad multiplication of *M. leprae* in neonatally thymectomized (Rees *et al.*, 1967) and congenitally athymic mice (Colston and Hilson, 1976) has substantiated the role of cells of T cell lineage in resistance to *M. leprae*. MHC restricted $CD4^+$ and $CD8^+$ T cells (Modlin *et al.*, 1988), CD1 restricted T cells (Sieling *et al.*, 1999) are involved. In the absence of effective adaptive immune response, *M. leprae* can multiply in macrophages to over 100 organisms per cell (Hagge *et al.*, 2004).

At the tuberculoid end, mostly $CD4^+$ helper cells with a $CD4^+/CD8^+$ ratio of 1.9:1 predominates, whereas in lepromatous lesion, the ratio observed is 0.6:1 (Modlin *et al.*, 1988). At the tuberculoid end, the immune response is driven more towards T_H1 type as evidenced by the presence of cytokines like interleukin 2 (IL2), Tumor necrosis factor-α (TNF-α) and Interferon-γ (IFN-γ). IL4, IL5 and IL10 predominate lepromatous end suggesting the role of T_H2 immune responses (Modlin, 1994; Yamamura *et al.*, 1991).

At the tuberculoid end, crosstalk between T cells and macrophages mediated by T_H1 type cytokines effectively contains the infections within granulomas (Modlin, 1994). Intracellular killing of *M. leprae* by activated macrophages is the major effector mechanism operating to eliminate infection within host. Studies have also shown the importance of reactive nitrogen intermediates for killing *M. leprae* (Adams *et al.*, 2000; Hagge *et al.*, 2007).

3.5.3 Reactions in leprosy

One of the conspicuous features of leprosy is the development of leprosy reactions, characterized by the acute inflammatory complications during the course of treated or untreated Hansen's disease (Scollard *et al.*, 2006). Two types of leprosy reactions have been defined. They are Type 1 reactions (T1R) or Reversal reaction and Type 2 reactions or Erythema Nodosum Leprosum (ENL).

a. Type 1 reactions : Type 1 reactions (T1R) are sometimes called reversal reaction (RR), borderline reactions, tuberculoid reactions or non-lepromatous lepra reactions. They usually occur in patients falling into borderline leprosy class (BT, BB and BL groups) (Scollard *et al.*, 2006). 30% of individuals with borderline leprosy are at risk of T1R (Van Brakel *et al.*, 1994). Clinically the skin lesions become acutely inflamed and oedematous and may ulcerate (Walker and Lockwood, 2006). The edges become more distinct than before making lesions stand out sharply from the surrounding normal skin. The lesions are often tender and even painful (Hastings, 1985). T1R often involve acute neuritis which if not treated leads to permanent loss of nerve function causing peripheral sensory and motor neuropathy (Walker and Lockwood, 2006). Pregnancy is one of the factors to have shown correlation with T1R (Lockwood and Sinha, 1999)

b. Type 2 reactions : Type 2 reactions are also known as lepromatous lepra reactions and erythema nodosum leprosum (ENL) (Hastings, 1985). These occur in multibacillary patients (BL and LL). It affects 50% of lepromatous and 10% of borderline lepromatous cases (Pocaterra *et al.*, 2006). Type 2 reactions are a systemic disorder affecting many organ systems. Clinically, small papule, 2-5mm, or larger nodules, which are painful and tender to touch, appear on the face, extremities and/or trunk, without predilection for existing lesions (Scollard *et al.*, 2006). The nodule may be superficial and stand out clearly from the skin, or may be placed deeply which is palpable rather than visible (Hastings, 1985). Type 2 reactions produce fever and malaise and some degree of neuritis leading to motor and sensory neuropathy (Scollard *et al.*, 2006). ENL reactions may also produce uveitis, neuritis, arthritis, dactylitis, lymphadenitis and orchitis. The recurrent inflammation of organs can lead to blindness and sterility (Walker and Lockwood, 2006).

3.6 Diagnosis of Leprosy

3.6.1 Clinical Diagnosis

Clinical diagnosis of leprosy is dependent upon recognition of disease symptoms and is therefore only possible once the disease has manifested (Walker and Lockwood, 2006). With the unavailability of standard culturing technique for *M. leprae* as for other human etiological agents, the diagnosis of leprosy has been based largely on clinical signs and symptoms.

Hypopigmented manifestation varies significantly among dark coloured and light coloured skin. Hypopigmentation is more conspicuous among dark skinned human and is hardly visible among people with light skin. Reddening of skin appears just the opposite (Hastings, 1985).

3.6.2 Laboratory Diagnosis

The laboratory diagnosis of leprosy is based on the demonstration of acid fast bacilli (AFB) in the tissue fluid collected under the skin from various anatomical sites or from the leprosy lesions. The preferred anatomical sites for slit skin smear preparation are the

two ear lobes, the right arm and the right knee or where the skin lesions or nodules are present. Multibacillary patients more typically demonstrate AFB positive smear from SSS; whereas, PB patients may be SSS negative. Slit skin smear is an important tool in classification of disease, grading the load of bacteria within the patient, monitoring the efficacy of antibacterial therapy and predicting the case of relapse and reinfection (WHO, 2003). However, the requirement of experienced personnel to obtain the slit skin scrapings and analysis limits its use in field settings.

3.6.3 Histological Diagnosis

Histological examination is regarded as the gold standard in diagnosis of leprosy (Scollard et al., 2006). A full thickness skin biopsy is taken from the advancing margin of an active lesion, fixed in neutral buffered formalin, embedded in paraffin and examined by an experienced pathologist for the host response in haematoxylin and eosin stained sections (Scollard *et al.*, 2006). Histological diagnosis helps in categorizing leprosy patients on a continuous spectrum of Ridley-Jopling classification.

3.6.4 Immuno Diagnosis

Tools to diagnose sub-clinical infection with *M. leprae* will aid to formulate early interventions to interrupt the chain of transmission (Sekar, 2007). The discovery of *M. leprae* specific antigenic Phenolic Glycolipid I (PGL-I) has been found useful in detecting IgM type immunoglobulins in the sera of leprosy patients (Moura *et al.*, 2008) by techniques such as ELISA (Douglas and Worth, 1984), dipstick assays (Buhrer *et al.*, 1998) and simple and fast lateral flow assay (Buhrer-Sekula *et al.*, 2003). Seropositivity was found to be 80-100% among lepromatous patients and 30-60% among the patients at the tubuerculoid end (Moura *et al.*, 2008). Asymptomatic, some PB and early stage *M. leprae* infection cannot be identified by the use of anti-PGL-I antibodies (Geluk *et al.*, 2008).

Many new antigens such as *M. leprae* Soluble antigen minus lipoarabinomannan (MLSA LAM), *M. leprae* cell wall antigen (MLCwA) and its fractionates have been studied with a view to develop them as skin test antigens (Sekar, 2007). The MLSA LAM is the cytosol derived and MLCwA is the cell-wall derived M. leprae fraction depleted of cross-reactive and immunomodulatory lipids (Weir *et al.*, 1999). The two

new antigens were developed as a tool to assay T cell response and lymphoproliferation in leprosy patients (Weir *et al.*, 1999). Particularly, the membrane fraction of *M. leprae* seems to be promising due to presence of many proteins unique to *M. leprae* (Araoz *et al.*, 2006; Brennan, 2000; Geluk *et al.*, 2008).

3.6.5 Molecular Diagnosis

Molecular methods have been employed in leprosy to detect *M. leprae* cells and monitor the effectiveness of chemotherapy (Lini *et al.*, 2009). PCR based methods have been developed for *M. leprae* diagnosis; however, field sites may have limited access the the technology, funding and expertise necessary to employ it. The PCR methods targeting the amplification of 16s rRNA (Bang *et al.*, 2009; Rudeeaneksin *et al.*, 2008), genes encoding 18 kDa protein (Williams *et al.*, 1990a), 36 kDa protein (Hartskeerl *et al.*, 1989), 65 kDa protein (Plikaytis *et al.*, 1990), leprosy serum reactive (Misra *et al.*, 1995) and repetitive DNA sequences of *M. leprae* (Banerjee *et al.*, 2008; Woods and Cole, 1989) have been studied for the specificity and sensitivity. Real time PCR based method targeting antigen 85B coding gene has been used as molecular diagnostic tool to detect *M. leprae* from frozen biopsy material (Martinez *et al.*, 2006).

3.7 Treatment

WHO recommended Multi-drug therapy (MDT) has contributed to the steep fall in prevalence of disease before and after 1982 (WHO, 1994). MDT regimen comprise of combination of drugs like rifampicin, clofazimine and dapsone (Table 5). MDT was recommended by WHO in order to potentially limit development of drug resistant *M. leprae*. Since 1998, the WHO also lowered the time period of drug administration to 12 months for MB patients and to 6 months for PB patients (Scollard *et al.*, 2006).

Table 5: MDT regimen for leprosy. Course of WHO recommended treatment in paucibacillary (PB) and multibacillary (MB) leprosy patients. (Source : Walker and Lockwood, 2006)

Type of leprosy	Drug treatment		Duration of treatment (months)
	Monthly supervised	Daily, self-administered	
Paucibacillary	Rifampicin 600mg	Dapsone 100mg	6
Multibacillary	Rifampicin 600 mg, Clofazimine 300 mg	Clofazimine 50 mg, Dapsone 100mg	12

Rifampicin is the primary anti-bacterial drug in use to treat leprosy. Second line drugs such as minocycline, ofloxacin, perfloxacin and clarithromycin have been studied as potential therapeutic agents in view of effectiveness and reduction of longer time span of treatment (Scollard et al., 2006).

In case of type 1 reaction or neuritis and silent neuropathy, treatment should be promptly started with corticosteroids if permanent nerve damage is to be avoided. Normally, 40-60 mg of prednisolone is administered initially and when the reaction is under control the dose is slowly tapered. Severe ENL is treated with prednisolone, clofazimine or thalidomide. Prednisolone is administered in the same way as for type 1 reaction. Clofazimine seems to be less effective in controlling ENL (Walker and Lockwood, 2006). A typical dose of 300-400 mg daily of thalidomide can effectively control ENL and may prevent recurrences (Walker and Lockwood, 2006). Thalidomide however is administered only under certain circumstances due to its teratogenecity and possible neurotoxicity (Jacobson and Krahenbuhl, 1999; Walker and Lockwood, 2006).

3.8 Transmission of Leprosy

Questions regarding how leprosy is transmitted among human hosts lack definite answers and is still under debate (Brandsma *et al.*, 2008). The problem is even more exacerbated by disputes on infectious source(s) (Matsuoka, 2009). Experimental infection with aerosols containing *M. leprae* in immune suppressed mice has implicated droplet infection (Rees and McDougall, 1977) as a possible mechanism of transmission.

This proposition is supported by studies demonstrating *M. leprae* in the nasal mucosa of lepromatous patients (de Wit *et al.*, 1993; Job *et al.*, 2008).

Contact transmission is another hypothesized mode of transmission of leprosy. Study has shown that long term close contacts of leprosy patients have an increased risk of developing leprosy as compared to non-contacts (Cardona-Castro *et al.*, 2009b). Contacts of lepromatous patient are at greater risk than those of non-lepromatous index case and in particular the household contacts (Duraes *et al.*). Skin has been particularly emphasized in the course of contact transmission. Skin trauma caused by any accidental injury (Ghorpade, 2009b), deliberate tattoing (Ghorpade, 2002, , 2009a), needle-stick injury sustained during taking biopsy for diagnostic purpose (Achilles *et al.*, 2004) have been implicated as a possible cause of developing leprosy in each of these cases.

Development of leprosy among human hosts who have had no contact with any leprosy patients indicate the possibility of environmental agents as a source of infection (Blake *et al.*, 1987). Observations support soil, vegetation, water, arthropods and armadillos (*Dasypus novemcinctus*) as nonhuman sources for leprosy (Blake *et al.*, 1987). Armadillos, in particular, are regarded as the only proven natural host to leprosy bacillus as demonstrated by the presence of bacillus in wild armadillos over a wide area of North America (Monot *et al.*, 2005). Moreover, association between armadillos contact and human cases of leprosy (Deps *et al.*, 2008) substantiates the role of armadillos in leprosy transmission. Armadillos exhibit full histopathological features of leprosy similar to human and can be detected in the same methods as used in human cases (Truman and Fine, 2010). Speculative examination on environmental sources has found the presence of viable *M. leprae* organisms in the soil (Lavania *et al.*, 2008). Similarly, *M. leprae* has been found to persist outside human body in various environmental conditions for a considerable period of time (Desikan and Sreevatsa, 1995). Intriguingly, a free living amoeba *Acanthamoeba castellani* is also found to be supporting the viability of the intracellular *M. leprae* at 20:1 multiplicity (Lahiri and Krahenbuhl, 2008) which substantiates its potential role in disease transmission.

3.9 Bacteriology of *M. leprae*

M. leprae measures 1-8 um long and 0.38 μm in width (Takade *et al.*, 2003). It is a straight or slightly curved rod-shaped organism with parallel sides and rounded ends (Hastings, 1985). It is a Gram positive, strongly acid fast, non-motile, non-spore forming microaerophilic, non-tuberculous mycobacteria. It is an obligate intracellular parasite, predominantly in macrophages, where the organisms commonly occur in clumps or 'globi' which may become very large containing hundreds of bacteria. In smaller clumps the organisms characteristically occur in parallel array resembling 'bundles of cigars'.

Attempts to grow *M. leprae* in vitro on artificial media have been futile but viability can be maintained in axenic cultures for few weeks (Truman and Krahenbuhl, 2001). As an alternative, propagation of *M. leprae* in animal models like the armadillo and normal, athymic and gene knock-out mice has provided basic resources for genetic, metabolic and antigenic studies of the bacillus (Scollard *et al.*, 2006). The organism grows very slowly with a generation time of 12.5 days (Jacobson and Krahenbuhl, 1999). Incubation at 37ºC rapidly reduces the viability of *M. leprae* (Truman and Krahenbuhl, 2001). Ability to catabolize carbon and nitrogen compound is severely limited in *M. leprae* in comparision to *M. tuberculosis* because there are fewer oxidoreductases, oxygenases and short chain alcohol dehydrogenases and their probable regulatory genes (Cole *et al.*, 2001a). The metabolic problem is further intensified by the loss of anaerobic and microaerophilic electron transport systems and that the aerobic respiratory chain is severely curtailed, making it impossible to generate ATP from the oxidation of NADH (Wheeler, 2001).

Electron microscopy studies *of M. leprae*'s cell envelope reveal the presence of electron translucent layer, a peptidoglycan layer and the plasma membrane (from outside to inside) (Takade *et al.*, 2003). The core cell wall consists of peptidoglycan, composed of chains of alternating N-acetylglucosamine and N-glycolylmuramate linked by peptide cross-bridge, which is linked to galactan layer by arabinogalactan. Three branched chains of arabinan are in turn linked to the galactan, forming, along with the peptidoglycan layer, an electron-dense zone around *M. leprae*. Mycolic acids are linked

to the termini of arabinan chains to form the inner leaflet of a pseudolipid bilayer. The outer leaflet is composed of a rich array of intercalating mycolic acids of trehalose monomycolates and mycoserosoic acids of phthiocerol dimycocerosates as well as phenolic glycolipds (PGL), forming the electron-transparent zone (Scollard *et al.*, 2006). The peptidoglycan layer in *M. leprae* is comparatively less thick than that in *M. tuberculosis* (Takade *et al.*, 2003).

3.10 Genome of *M. leprae*

The complete genome sequence of an *M. leprae* strain originally isolated from an MB patient from Tamil Nadu of India was decoded in 2001 (Eiglmeier *et al.*, 2001). It was revealed that the genome sequence of *M. leprae* contained 3268203 base pairs (bp) and had an average G+C content of 57.8%. There is a single circular chromosome and no plasmids (Eiglmeier *et al.*, 2001). Comparative genome study revealed the values to be much lower than that of the *M. tuberculosis* genome which consists of 4411532 bp and an average G+C content of 65.6%. Only 49.5% of the genome accounted for protein-coding genes while 27% accounted for recognizable pseuodogenes, the functional counterparts of which could be detected in *M. tuberculosis* genome. The remaining 23.5% of the genome does not account for any protein coding gene and may possibly be the regulatory sequences or the gene remnants mutated beyond recognition. There are 1604 genes capable of coding a protein in *M. leprae*, of which 1439 genes are common in *M. tuberculosis* as well. Of the 165 genes unique to *M. leprae*, 29 genes could be assigned functions while 136 genes, which show no similarity to know genes, may also represent pseudogenes owing to their shorter length and occurrence in regions of low gene density (Cole *et al.*, 2001a).

Table 6: Comparison of genome features. Comparison of different genomic features of *M. leprae* and the member of the same genus *M. tuberculosis*. Extensive pseudogenes and considerable genome downsizing could be observed in *M. leprae*. * indicates number of pseudogenes excluding IS elements. (Source : Cole *et al.*, 2001a)

Feature	*M. leprae*	*M. tuberculosis*
Genome size (bp)	3,268,203	4,411,532
G+C (%)	57.79	65.61
Protein Coding (%)	49.5	90.8
Protein-coding genes (no.)	1604	3959
Pseuodogenes	1,116	6*
Gene density (bp per gene)	2,037	1,114
Average gene length (bp)	1,011	1,012
Average unknown gene length (bp)	338	653

Recently, the genome of a second strain of *M. leprae* from Brazil (Br4923) has been sequenced and found to contain 3268071 bp which is 141 bp smaller than that of the *M. leprae* TN strain (Monot *et al.*, 2009). When compared with TN strain, the genome did not show any evidence of DNA inversions, translocations or duplications and no transposition or amplification of insertion sequence elements or the four families of dispersed repeats. The two genomes differed from each other at 194 polymorphic sites, comprising 155 Single nucleotide polymorphisms (SNPs), 31 Variable Number of Tandem Repeats (VNTRs) and 8 insertion-deletion (indel) events. The distribution of SNPs were 52 in genes encoding proteins, 39 in pseudogenes, 26 in non-coding regions and 38 in dispersed repeats. The majority of the VNTRs (23 of 31 VNTRs) in Brazilian strain Br4923 were found to be shorter than those in TN strain. TN strain consisted of relatively higher repeat copy numbers at these VNTR sites. Indels occurred in repetitive elements (6 of 8 indels), one in an intergenic regions and the other in the gene *ML0825c* (Monot *et al.*, 2009).

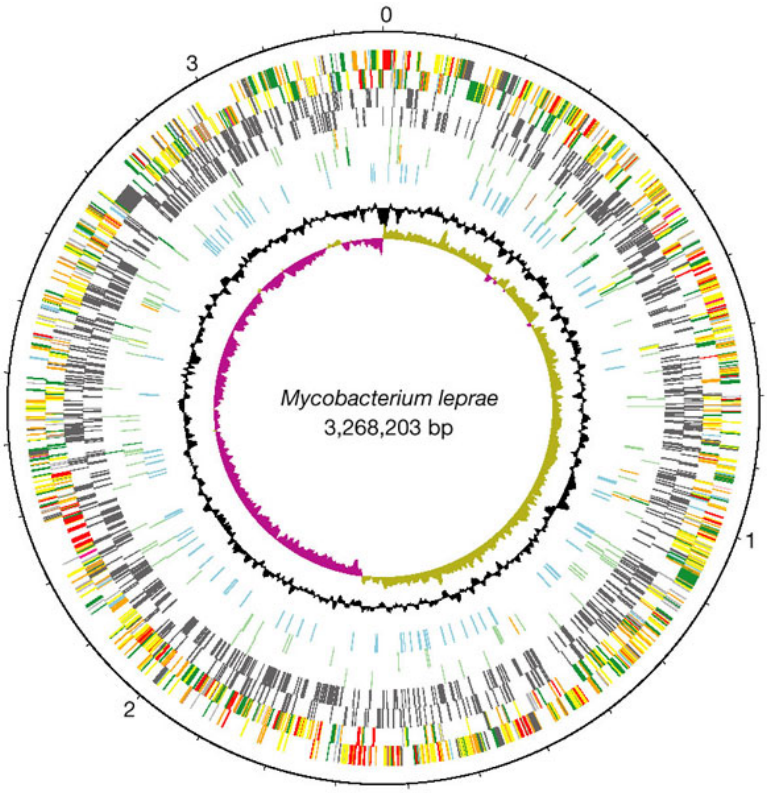


Fig 2. Circular genome map showing the position and orientation of known genes, pseudogenes and repetitive sequences. From the outside: circles 1 and 2 (clockwise and anticlockwise) genes on the - and + strands, respectively; circles 3 and 4, pseudogenes; 5 and 6, *M. leprae* specific genes; 7, repeat sequences; 8, G+C content; 9, G/C bias (G+C)/(G-C). Genes are colour coded using the following functional categories8: lipid metabolism (dark grey); intermediary metabolism and respiration (yellow); information pathways (red); regulatory proteins (light blue); conserved hypothetical proteins (orange); proteins of unknown function (light green); insertion sequences and phage related functions (pink); stable RNAs (dark blue); cell wall and cell processes (green); PE and PPE protein families (magenta); virulence, detoxification, adaptation (brown). (Source : Cole *et al.*, 2001a).

Comparative genome analysis of four strains of *M. leprae* from diverse geographical regions viz. TN strain from India, Br4923 from Brazil, NHDP63 from the United States

and Thai-53 from Thailand has shown remarkably little genomic diversity among the strains (Monot *et al.*, 2009). The strains share 99.995% similarity. This further validates the assumption that *M. leprae* is a single clone descendant and has passed through a recent evolutionary bottleneck (Maiden, 2009; Monot *et al.*, 2009), .

3.10.1 Reductive evolution and its consequences in *M. leprae*

Comparison of genome sequences of *M. leprae* (3.3 Mb) and its close relative *M. tuberculosis* (4.4 Mb) has documented extensive genome downsizing or reductive evolution. Genome downsizing has been speculated to result from extensive recombination events between dispersed repetitive sequences (Cole *et al.*, 2001a). The consequences of genome downsizing in *M. leprae* is the elimination of several metabolic pathways thereby leading to its survival in intracellular niche utilizing the host factors. However, functional genes involved in gene regulation, metabolism and modification of fatty acids and polyketides, cell envelope synthesis and transport of metabolites have remained intact (Scollard *et al.*, 2006; Vissa and Brennan, 2001) . Some 2000 genes have been assumed lost since diverging from the last common mycobacterial ancestor (Cole *et al.*, 2001a)

3.10.2 Repetitive DNA

Sequences when present in multiple copies within a genome are known as repetitive DNA (Cox and Mirkin, 1997; Fowler *et al.*, 1988) . Repetitive DNA is a prominent feature of eukaryotic genome occurring as homopolymeric tracts of a single nucleotide type [poly(A), poly(G), poly (T), poly(C)] or of a small numbers of several multimeric classes of repeats built from identical units (homogenous repeats), mixed units (heterogenous repeats) or degenerate repeat sequence motifs (Jeffreys *et al.*, 1985).

There are two general types of repetitive DNA; Moderately repetitive DNA and Highly repetitive DNA. Moderately repetitive DNA has relatively shorter repeat sequences and occur typically 10-1000x in a genome. Distribution is random. Highly repetitie DNA has very short (<100bp) repeat sequences and occur many thousands of times in the genome often organized as long tandem repeats.

Repetitive sequences with variable structure, size and distribution have also been identified in bacteria such as member of the *Enterobacteriaceae*, *Haemophilus influenza*, *Streptomyces*, *Neisseria gonorrhoes*, *Thiobacillus ferroxidans*, *Rhizobium* and *Agrobacterium* species, *Bordetella pertusis* and *Mycoplasma pneumoniae* (Gilson *et al.*, 1987). The distribution of the repetitive sequences may be either localized being from an unequal crossing over event (Anderson and Roth, 1977) or dispersed throughout the genome (Woods and Cole, 1990). Approximately 2% of the *M. leprae* genome consist of repetitive DNA (Cole *et al.*, 2001b).

Dispersed repeats are one of the two principal forms of repetitive DNA in a bacterial genome. Dispersed repetitive sequences can correspond to duplicated genes or to mobile genetic elements present in several copies, like insertion sequences (Hatfull and Jacobs, 2000). In *M. leprae*, there are more than 26 extinct Insertion sequence (IS) elements together with four families of dispersed repeats, present in five copies or more, RLEP (37 copies), REPLEP (15 copies), LEPREP (8 copies) and LEPRPT (5 copies) (Cole *et al.*, 2001b).

RLEP elements have been utilized to enumerate *M. leprae* from various tissues by techniques such as real-time PCR (Truman *et al.*, 2008). Structurally, the RLEP elements share a 545 bp invariable central domain flanked by a 100 bp left-end and a 44 bp right-end, sometimes associated with a 47 bp extension. The presence of left and right-ends are variable (Woods and Cole, 1990).

3.10.2.1 Tandem Repeats in *M. leprae*

Tandem repeats (TR), also known as satellite DNA, are the repeat sequences aligned in a head to tail fashion with each other and without gaps in between (Clark and Russell, 2005). TRs can be relatively simple, such as multiple repetitions of di- or tri-nucleotide sequences or more complex such as the tandem duplication of large chromosomal segments. TRs are usually classified among satellites (spanning megabases of DNA), minisatellites (repeat units in the range 6-100bp, spanning hundreds of base pairs) and microsatellites (repeat units in the range 1-5 bp spanning a few tens of nucleotides) (Le Fleche *et al.*, 2001). When the repetitive core sequence are shorter ranging from 1-5bp,

the tandem repeats have been called Simple sequence repeats (SSR) (Jacob *et al.*, 1991), microsatellite (Litt and Luty, 1989) or Short tandem repeat (STR) (Edwards *et al.*, 1991). A polymorphism in the number of repeats when the repeating unit is longer (in the range of 10-60 nucleotides) is called a variable number of tandem repeats (VNTR) (Hartl and Jones, 2009).

Tandem repeats can occur within a regulatory region of a gene or within a coding region of a gene thereby contributing to phenotypic variation in bacteria (Le Fleche *et al.*, 2001). When it occurs at a regulatory region, it can constitute an on/off switch of gene expression at the transcriptional level. Similarly, when it occurs in coding regions with repeat units length not a multiple of three, it can induce a reversible premature end of translation when a mutation changes the number of repeats. But when the TRs occur in coding region with repeat unit length in a multiple of three, variation in the number of copies can modify the gene product itself (Le Fleche *et al.*, 2001). Repeat variability can change the physical integrity of functional DNA domains, whereas open reading frames can be disrupted or otherwise modified (van Belkum *et al.*, 1998). In bacteria many of the TRs appear to be situated within coding sequences. As such, one important functional effect concerns the mediation of phase variation through the loss or gain of one or several repeats (Yeramian and Buc, 1999). *H. influenzae* exemplify this particular feature by undergoing phenotypic variation in surface molecules interacting with the host constituents (Robertson and Meyer, 1992) thereby conferring it an advantage to survive in hostile environments.

3.10.2.1.1 Mechanisms leading to repeat variability

Among the diverse mechanisms leading to repeat variability, two have been identified as key for VNTR loci (van Belkum *et al.*, 1998). Recombination can be a major mechanism explaining VNTR behavior, but so called Slipped-strand mispairing (SSM) is the phenomenon most frequently held responsible for variability in repeat numbers (van Belkum, 1999). Length changes in microsatellite are generally thought to arise from replication slippage-that is, transient dissociation of the replicating DNA strands followed by misaligned reassociation. When the nascent strand realigns out of register, renewed replication will lead to the insertion or deletion of repeat units relative to the

template strand. Slippage involves DNA polymerase pausing, during which the polymerase dissociates from the DNA. On dissociation, only the terminal portion of the newly synthesized strand separates from the template and subsequently anneals to another repeat unit (Ellegren, 2004). Recombination events more specifically define the repeat variability in minisatellite region. Recombination-like processes that involve unequal crossover or gene conversion introduce mutations in the larger minisatellites sequences (Berg *et al.*, 2003).

3.10.2.1.2 Molecular analysis of TRs

Repetitive DNA show characteristic physical properties. The detection of repetitive DNA in eukaryotic chromosomes was initially based on aberrant density analysis (Britten and Kohne, 1968). When whole genomic DNA was subjected to density gradient centrifugation, repetitive DNA lagged behind the bulk of DNA and presented as satellite fractions due to differences in thermodynamic stability and reassociation kinetics (Britten and Kohne, 1968).

Initially, characterization of repeat variability was based on Southern hybridization techniques with DNA probes recognizing the repeat consensus motif (van Belkum *et al.*, 1998). With the advent of PCR, it has become easier. In this method, PCR primers bordering the SSR regions are constructed. After the amplification of the loci, an electrophoretic technique is applied to separate the amplicons based on their sizes. Based on the number of repeat copy numbers at particular loci, the amplicons will show up as differently sized bands within the gel. Thus the polymorphisms at these loci could be detected. The regions bordering the repeats are generally well conserved within the species (van Belkum *et al.*, 1998).

More recently, multiplex PCR amplification of four to five TR loci has been designed to increase throughput (Kimura *et al.*, 2009). Further, the use of fluorescent tagged primers for use in DNA sequence analyzers has made possible the exact estimation of amplicon sizes. Estimating the exact amplicon sizes is necessary to compare with the data from similar studies done elsewhere (Kimura *et al.*, 2009).

3.11 Utility of VNTRs for typing *M. leprae* strains

M. leprae is uncultivable in any of the known bacteriological media. So, classical phenotypic methods are not available for typing *M. leprae.* This has warranted the switch for genotypic methods to differentiate between *M. leprae* strains. Genotyping of bacterial pathogens can enhance the ability of identifying the source of infection or the time frame of different outbreaks for better understanding of disease transmission (Supply *et al.*, 2000). VNTRs have been employed in molecular epidemiological studies of various bacterial pathogens like *M. tuberculosis* complex (Skuce *et al.*, 2002), *M. caprae* (Prodinger *et al.*, 2005), *Yersinia pestis* (Klevytska *et al.*, 2001), *Helicobacter pylori* (Marshall *et al.*, 1996), *Haemophilus influenzae* (van Belkum *et al.*, 1997) and *B. anthracis* (Keim *et al.*, 2000).

Methods based on molecular markers have been developed to elucidate the phylogenetic relationships between the bacterial isolates (Wang *et al.*, 2009). In essence, molecular markers with low variability are useful to establish phylogenetic relationship among bacterial isolates evolved over longer time spans and highly variable markers are more useful to resolve closely related isolates for the purpose of outbreak investigation and disease surveillance (Wang *et al.*, 2009).

Restriction fragment length polymorphism (RFLP) studies have been ineffective in typing *M. leprae* isolates due to extreme homology among the genomes of *M. leprae* isolates from diverse origins, including leprosy patients, armadillos and mangabey monkeys (Clark-Curtiss and Walsh, 1989; Monot *et al.*, 2009). Variation in the RFLP patterns could not be demonstrated despite the isolates being obtained from geographically distinct areas (Williams *et al.*, 1990b). Similarly, sequencing studies of the intergenic spacer region between 16S and 23S rRNA genes have not been useful to differentiate among *M. leprae* isolates owing to the absence of variations within the sequence (de Wit and Klatser, 1994). Subsequently, the *polA* gene, encoding DNA polymerase, was identified to be flanked by two inverted copies of repeat sequence RLEP, the copy number of which was found to vary among different *M. leprae* isolates (Fsihi and Cole, 1995).

With the availability of *M. leprae* genome sequence, DNA repeat loci composed of TTC elements was identified (Shin *et al.*, 2000). The study revealed variation at the loci with the repeat number ranging from 10 to 37 among *M. leprae* strains isolated from 34 multibacillary patients from Cebu, Philippines. It was observed that among the Philippine population, 24 and 25 TTC repeats were more common than others. TTC repeats were not found in *M. tuberculosis, M. avium, M. marinum* or human tissues, and thus substantiate its specificity and usefulness to distinguish between strains of *M. leprae* for epidemiological investigations of leprosy (Shin *et al.*, 2000). However, typing schemes based on single microsatellite loci tend to severely limit the discriminatory power (Matsuoka *et al.*, 2004; Truman *et al.*, 2004). Similar studies of strain characterization based on TTC polymorphism have been conducted in villages of North Maluku where *M. leprae* infections are endemic (Matsuoka *et al.*, 2004). The study found differences in TTC copy numbers among isolates from father and son and also between a son under treatment and the other two brothers. The results thus indicate the possibility of other infectious sources other than a single multibacillary case of leprosy within family members. A study conducted in Nepal (Pandey, 2005) found wide variation in copy numbers of TTC repeats among 118 Nepalese isolates. Repeat numbers ranged from 3 to 30 with 13 and 15 being the most common isolates.

The *rpoT* gene containing 6bp tandem repeats has also been studied among 51 *M. leprae* isolates from 12 geographically distinct regions of the world (Matsuoka *et al.*, 2000). The study revealed that isolates from Japan (except Okinawa) and Korea belonged to one group with 3 repeats while those from Brazil, Haiti and Okinawa in Japan belonged to a second genotype with repeat number four. Such markers are useful to trace the origins of the *M. leprae* geographically (Matsuoka *et al.*, 2000).

With an objective to characterize additional polymorphic sites, Groathouse *et al.* (2004), conducted a PCR based fragment length polymorphism study of 11 short tandem repeat loci among four clinical isolates of *M. leprae* propagated in armadillo host (Groathouse *et al.*, 2004) . The study showed all the five minisatellite loci studied (6-7, 12-5, 18-5, 21-3, 27-5) exhibited polymorphism due to VNTR. Of the 6 microsatellite loci studied, five of them were shown to be polymorphic. (C)20, (AT)17, (TA)18 and (GTA)9 loci

were resolved on agarose gel and polymorphism could be detected due to VNTR. (AGA)20 locus which was previously known as (TTC)21 locus was also studied and found to contain 10, 12 and 14 repeats among four isolates. (CG)6 locus on the contrary was not polymorphic. (AC)9 was also not polymorphic based on the agarose gel analysis. With the characterization of 9 polymorphic loci, the study aimed to build a minimum set of typing markers for genotyping *M. leprae* strains (Groathouse *et al.*, 2004).

Truman *et al.* (2004) investigated further into the genomic diversity and the stability of several VNTRs. Four of the five loci studied had previously been shown polymorphic and the study further validated the use of these markers in strain differentiation: GAA, AT17, GTA and TA18. However, C16G8 loci did not offer advantage towards the repertoire of molecular markers suitable for typing *M. leprae*. Allelic diversity was recorded to be highest for the TA18 locus and the least for GTA locus when analyzed among 12 *M. leprae* isolates derived from patients in different regions of United States, Brazil, Mexico and the Philippines. When checked for the stability of the genotype by passaging through nude mice and armadillos, relatively little variation was observed between the loci. However, variation was more common at the TA18 locus after long-term expansion in armadillos.

A study involving 33 leprosy patients in Hyderabad, India demostrated extensive diversity at the three microsatellite loci studied: TTC, AGT and TA (Young *et al.*, 2004). In the same study, a case of multicase household showed no divergence in two of the three loci studied. However, genetic diversity was prominent among *M. leprae* population isolated from peripheral nerve biopsy specimen within the same individual when compared with specimen from skin tissue. Such discrepancies have been proposed to arise from selective advantage conferred to heterogeneous population thriving in neural tissues (Young *et al.*, 2004).

A systematic study of 44 STR loci including 33 microsatellite and 11 minisatellite inferred the applicability of 9 polymorphic loci for identifying genotypes according to discriminatory capacity, stability and reproducibility (Zhang *et al.*, 2005). The nine

polymorphic microsatellite loci were (AC)9, (AC)8b, (AC)8a, (TA)10, (AT)17, (AT)15, (GTA)9, (TA)18 and (AGA)20. The study also validated polymorphisms observed previously at minisatellite loci such as 6-3a, 6-7, 12-5, 18-8, 21-3 and 27-5 (Zhang *et al.*, 2005).

With the characterization of tandem repeat loci and their proven usefulness in distinguishing between isolates of *M. leprae*, several studies have been undertaken to elucidate possible relationships between strains and to detect short range transmission events. A MLVA study based on 8 STR loci recruiting 68 patients in Quibei County, Yunan province of China identified several clusters in which *M. leprae* isolates shared similar VNTR patterns (Weng *et al.*, 2007). Studies on multicase families revealed closely matched intrafamiliar VNTR profiles. The VNTR patterns existing among multicase families could also be detected in the same or an adjacent township which signifies the use of VNTR strain typing to identify and detect short range transmission (Weng *et al.*, 2007). A preliminary study on Chinese populations had concluded molecular markers of STR loci like *rpoT*, (GGT)5, (AC)8b, 12-5, 21-3, 18-8 and 23-3 as insufficient for strain typing due to little polymorphism at these loci (Weng *et al.*, 2006).

Another Chinese study documented variations in the STR loci studied among *M. leprae* from different patients and also within the same patient (Young *et al.*, 2008). The latter findings led them to question on suitability of the loci as a general tool for population based studies or to distinguish between relapse and reinfection.

Methodological breakthrough in multiple-locus VNTR analysis (MLVA) of *M. leprae* has been provided by a study conducted by in terms of rapidity with which multiple VNTR loci could be analyzed (Kimura *et al.*, 2009). The primer pairs for 15 different VNTR loci were incorporated as a combination of fours or fives and a multiplex PCR was carried out. The forward primers had their 5'-end tagged with one of four fluorescent dyes: 6-FAM, VIC, NED, PET. Fragment length analysis (FLA) uses a DNA sequencer to detect these fluorescent tags for studying diversity of alleles at VNTR loci. The method also has lower cost than full sequencing and can use minimum

amounts of clinical specimen (Kimura *et al.*, 2009). Molecular epidemiological studies were also carried out with leprosy patients of Cebu, Philippines based on single nucleotide polymorphism (SNP) analysis and VNTR analysis by FLA technique (Sakamuri *et al.*, 2009b). The findings demonstrated that the majority of the strain circulating within Cebuano population belonged to SNP type1 and only 16% to SNP type3. The VNTR profiles could identify *M. leprae* genotype associations for patients with known epidemiological links such as in multicase families. The study also speculates of mixed infection when it found two distinct alleles at several VNTR loci by FLA for the sample collected from same individual

In a study conducted in South India (Shinde *et al.*, 2009), alleles for (AC)8b, (GGT)5, 6-3a (*rpoT*), 21-3, 27-5 and 23-3 were conserved in two study populations. In the same study, 14 of 15 STR loci did not show intra-patient variation indicating the suitability of STR strain typing for assessing short range transmission chains. In a study conducted in Thailand (Srisungnam *et al.*, 2009) based on 14 loci, microsatellite loci (GAA)21, (AT)17 were shown to be highly polymorphic followed by (GTA)9, (AC)8a, (AC)8b and (AC)9 23-3 was stale without polymorphism. 6-7, 21-3 and 27-5 were moderately polymorphic among studied samples. The study also suggests common infection source(s) based on closely matched VNTR profiles found among household members of two multicase families. Similar findings were also observed Chinese population (Xing *et al.*, 2009) and (GTA)9 and (TTC)21 loci were found to be hyper variable in this case.

Unlike RFLP and other classical methods, VNTR based methods provide data in a definitive numerical format which is comparably easier for analyses in global molecular epidemiology of infectious agents (Frothingham and Meeker-O'Connell, 1998). For this study, three new techniques will be tested for use in Nepal. Slit skin scrapings samples will be used for DNA isolation, which is less invasive than accessing a skin biopsy for VNTR analysis. In addition, this study is based on multiplex PCR increasing efficiency in time and the number of individual PCR reactions to be performed. Thirdly, size estimation of amplicons will be performed by FLA, a more precise and globally standardized method for VNTR typing.

Chapter 4
Material and Methods

4.1 Materials

A complete list of reagents, chemicals, equipments, glasswares and miscellaneous material required for the study is given in Appendix I.

4.2 Methodology

4.2.1 Study Site

Anandaban Leprosy Hospital and its weekly clinic at Patan Hospital were selected for the recruitment of leprosy patients for the study. Sample collection was carried out from September 2008 to March 2010. Sample analysis was performed at Mycobacterial Research Laboratory (MRL), Anandaban Hospital and the National Forensic Science Laboratory, Khumaltar.

4.2.2 Study Sample

Slit skin scrapings from various anatomical sites were collected by qualified hospital technicians. Various anatomical sites included the two earlobes, right arm, right thigh, lesions or nodules when present. Detailed information of the patient regarding age, sex, location (zone, district, VDC/municipality), bacillary index (BI), household contacts, clinical classification and hospital reference number were recorded. Patient information is provided at Table 8.

4.2.3 Slit-skin scraping collection

Anatomical sites from where the scraping was to be collected were disinfected by alcohol swab. With the help of a sterile stainless scalpel blade, a cut was made at the margin of the lesion, when present. With the lateral aspect of the scalpel, tissue fluid was collected. The first swipe was used for the slit skin smear clinical test. Material collected from second swipe was used for the study (WHO, 2003). The scalpel containing tissue fluid was then transferred to a labeled microcentrifuge tube containing 0.5-1ml of 70% alcohol (Bengal Chemicals and Pharmaceuticals Ltd., India). The

scalpel was soaked in 70% alcohol until it was transported to the laboratory. DNA extraction from the samples was carried out within 24-48 hrs after the collection. The samples were discarded if rust appeared.

4.2.4 DNA preparation from Slit-skin scraping

The microcentrifuge tube was briefly vortexed to suspend tissue from the blade in the alcohol. With a pair of forceps sterilized over flame, the blade was picked up and disposed into a biohazard sharps container. Particulate material was collected by centrifugation at 12000g for 20mins at room temperature (RT) (Centrifuge 5415C, Eppendorf, Germany). Sediment was then resuspended in 0.5ml Phosphate buffered saline (pH 7.4) (See Appendix II) and held for 1 hour at RT to rehydrate the skin scrapings. The particulate fraction was collected by centrifugation at 12000g for 20 min at RT. Sediment was resuspended in 50µl of lysis buffer (pH 8.0) (See Appendix II). It was then incubated at 60°C overnight in water bath (Grant, UK). After incubation, it was heated at 97°C for 10min in heat block (DB2A Techne, UK). It was then cooled to RT and briefly centrifuged. The DNA extract was stored at 4°C or -20°C for prolonged preservation (WHO, 2009a).

4.2.5 Spectrophotometric determination of DNA concentration and purity

5µl of DNA extract was diluted in 500µl of distilled water. The diluted samples were then used to measure the concentration of DNA against a distilled water blank in a spectrophotometer (Biophotometer, Eppendorf, Germany). $A_{260/280}$ ratio was calculated to determine purity of the DNA preparation (Sambrook *et al.*, 2001).

4.2.6 Multiplex PCR for VNTR loci

Eighteen different VNTR loci were studied. Primers for all the loci were kindly provided by Dr. Varalakshmi D. Vissa of Colorado State University, Fort Collins, Colorado, USA. The forward primer of each VNTR primer pairs was tagged with either of four fluorescent dyes: 6-FAM, NED, PET or VIC. Multiplex PCR was performed in four different combinations of primer pairs as shown in Table 7.

Table 7: Multiplex STR primers in four different combinations.

Multiplex PCR Combination Number	Locus[a]	Forward Primer	Tm (ºC)	Reverse Primer	Tm (ºC)	Amplicon size[b] (bp)
Combination 1	(AC)8b	VIC-GCCCACTTACCTCAACCAAC	65.4	CCTATAACGGCACTCAGTCCA	61.4	390
	(GTA)9	NED-AGCCTTAGTCGCGCAGATG	62.6	TCCGCTGTCCGTCCGCTGA	68.8	307
	(GGT)5	6FAM-GCAGCGGTGTAACAGCATAGC	68.4	TGTCTGCCTTGCGAAACGGTC	65.5	242
	(AT)17	PET-TCTCCAACATGCTGCGACA	62.1	GTACAGCGGCCTGATCGAA	60.0	181
	6-3a[C] (*rpoT*)	VIC-ATGCCGAACCGGACCTCGACGTTGA	60.0	TCGTCTTCGAGGTCGTCGAGA	59.5	91
Combination 2	21-3	6FAM-GAATCTGACCTTTCGGAAATG	56.8	CGATGCAGCTTCCTACGG	60.7	312
	(AC)9	NED-AGCGCCCGTTGTCGATAGA	63.9	GACTGGATGTCGGCACCCC	65.5	236
	(AT)15	PET-CAATATGCGGGTTGGCGCTTCTG	66.2	CCGTCTGGCTCGATGGCTGGATTC	68.6	168
	(AC)8a[C]	VIC-GTGTTACGCGGAACCAGGCA	65.5	CCATCTGTTGGTACTACTGA	53.5	124
	ML01	VIC-GCCTGGCTGATCTGTAGAG	53	GCTAACGGCCAAGGTTAC	50	272
Combination 3	27-5	6FAM-ATTGAGCAGATGGCCGGTC	62.8	AGCAGTCGGCACGCCCTT	67.8	327
	6-7	VIC-GCCATCGTTGTCGGTTCATC	61.5	CGGAGGAGGTGGGTACGGT	66.1	268
	(TA)18	NED-CGTGCGTCGTGTGTAGGC	63.9	GACGTGGCAACATCGAAGTT	61.0	230
	(GAA)21	PET-CTACAGGGGGCACTTAGCTC	62.1	GGACCTAAACCATCCCGTTTT	60.4	201
Combination 4	18-8	PET-GCCCGTCTATCCGCATCAA	62.5	GCAAAGATCAGCACGCCAAT	61.8	348
	12-5	VIC-CTGGTCCACTTGCGGTACGAC	65.1	GGAGAAGGAGGCCGAATACA	61.4	289
	23-3	6FAM-CCGAAGCCCTGGACGAAG	63.1	GCCGTAAATCCGCTCCC	56.0	243
	(TA)10[C]	PET-TAGATTCAAACGACCATGCA	60.0	TGATAATCACGTGTTTCCGC	60.0	185

a tandem repeat locus nomenclature per Groathouse *et al.JCM*
b amplicon sizes per the sequenced *M. leprae* TN strain
c primer sequences per Zhang *et. al.JCM*
VIC, NED, 6FAM and PET refers to the 5' Fluorochrome.

4.2.6.1 Primer preparation

The lyophilized primers were reconstituted in 1x Tris-EDTA (TE) buffer (pH 8.0) (See Appendix II) to a working concentration of 10μM. This served as an original primer stock. As per the table, primer mix for different combinations was prepared. 20μl of each forward primer from a combination set was added into an eppendorf tube, so that the final concentration of each primer was at 2μM (Working stock). In case of combination 3 and 4 (which has 4 VNTR loci), 20μl TE buffer (pH 8.0) was added to obtain final concentration at 2μM. Similarly, reverse primer mix was prepared. So prepared primer mix was used for multiplex PCR. Extra original primer and working stock mix was stored at -20°C until use.

4.2.6.2 Multiplex PCR protocol

A biological safety cabinet class II (BH12, Labcaire System Ltd., UK) was thoroughly disinfected with 70% alcohol and illuminated with UV light for 10mins for sterilization. 2x Qiagen multiplex PCR master mix (Qiagen, Germany), primer mix and Q-solution (Qiagen, Germany) were thawed inside the hood. All the solutions were mixed by vortexing. Template DNA was thawed outside hood.

PCR reaction master-mix was prepared as following :

Component	**Volume/Reaction**
Qiagen Mutiplex PCR mastermix (2x)	12μl
Forward Primer mix (2 μM) (Working stock)	2μl
Reverse Primer mix (2 μM) (Working stock)	2μl
Q-solution	2μl
Total	18μl

A master reaction mix according to the number of samples was thoroughly mixed and 18 μl of the mix was dispensed into each PCR tube. 2μl of template DNA was added. The PCR tubes were then placed in a thermal cycler (T3 Thermocycler, Biometra, Germany) and amplification started according to the following cycling parameters.

Initial activation step : 15 mins at 95°C

Denaturation : 30 sec at 94°C
Annealing : 90 sec at 60°C
Extension : 90 sec at 72°C
} 40 times

Final Extenstion : 10 min at 72°C

4.2.7 Polyacrylamide gel electrophoresis (PAGE)

The resulting PCR products were then subjected to polyacrylamide gel electrophoresis (8%) (Biometra, Germany) and DNA fragment analysis in ABI310 Genetic Analyzer (Applied BioSystems, USA).

8% polyacrylamide gel was prepared according to Sambrook and Russell (2001) (Appendix I). 10µl of amplified DNA mixed with 5µl of Bromo Phenol blue (BPB, Sigma, USA) was electophoresced at 100V for initial 5 mins and then 30V for 16 hours. The gel was stained with ethidium bromide solution (Sigma, USA) and the bands were visualized in the gel-doc (Bio Doc, UVP Transilluminator, UVP-upland, USA). A 100bp DNA ladder (Banglore Genei, India) were run simultaneously as the standards.

4.2.8 DNA fragment analysis

The amplified DNA products were diluted 30-50 fold in distilled water (Qualigens, India). 1µl of diluted sample was then mixed with 12µl deionized formamide (Applied Biosystems, USA) and 0.3µl LIZ-500 DNA standard (Applied Biosystems, USA). The sample was denatured at 94°C for 5 min and immediately cooled in the freezer. The samples were then injected into a capillary tube (36cm, POP4 polymer) by applying an injection voltage of 1kV for 5 sec. The capillary electrophoresis was run at a voltage of 15kV at 60°C in ABI 310 Genetic analyzer (Applied Biosystems, USA) (Kimura *et al.*, 2009). A standard *M. leprae* NHDP63 (National Hansen's Disease Program, USA) strain was also run along with the samples of each batch to facilitate comparision. Following the separation, the electropherograms were visualized and analyzed using GeneMapper ver 3.2 software (Applied Biosystems, USA) to determine the major allele for each VNTR locus in each multiplex PCR combination.

4.2.9 Data Storage and Analysis

Data were entered and stored as a worksheet in Excel (Version 2003, Microsoft). Similary, the results on DNA quantification, DNA purity, amplicon sizes in bp and allele calls were stored in Excel worksheet along with date and sample identity.

The images from PAGE were stored as .jpeg files. The results from DNA fragment analysis performed by GeneMapper ver. 3.2 (Applied Biosystems, USA) were stored as .fsa file and the electropherograms were produced as .pdf files.

The web program, Microsatellite-toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit), was used to calculate the diversity index, allele frequency and to compute matches

Chapter 5
Results

In the present study, typing of *M. leprae* strains isolated from slit-skin scrapings (SSS) was performed. The strain typing method was based on multiplex-PCR amplification of 18 different polymorphic VNTR loci which constituted both minisatellite and microsatellite markers (Gillis *et al.*, 2009; Kimura *et al.*, 2009).

5.1 Study Sample

Of the total leprosy patients visiting Anandaban Hospital and its weekly Leprosy Clinic at Patan Hospital, samples were collected from 168 patients. The 168 pateints were selected on the basis of their positive BI and being MB patient. Out of this, 40 MB patients were selected with average BI $\geq$ 1+. From these 40 patients, a single SSS sample was selected for the study based on high BI at the particular site and higher DNA purity as measured by $A_{260/280}$ value. The selected samples had BI $\geq$ 2+. SSS samples were selected from the site of lesion, nodules or the routine sites. Basic patient information for the 40 selected study samples are depicted in Table 8.

Among the 40 MB leprosy patients: 31 patients were LL, 8 patients were BL and 1 patient was BB. MB patients were selected primarily because the samples from the lesions have higher chances of having abundant *M. leprae* cells from which to extract DNA. The BI of individual patients ranged from 1+ to 4.25+ and that of processed samples ranged from 3+ to 6+. The average BI of processed samples was calculated to be 4.1+.

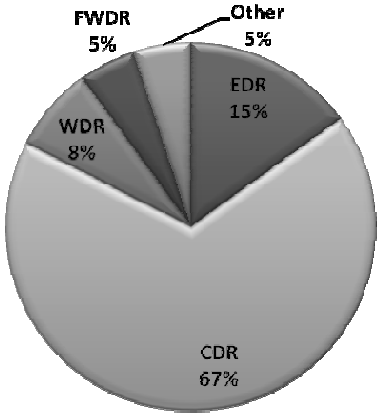
Table 8: Patient Sample Demographics and Clinical information. Basic information including sample number, age, sex, district, classification of disease, leprosy reaction status, average BI of the patient and the BI of the selected sample for 40 studied samples; S.No. = Sample number; n/a = not available; NR = No reaction

S.No.	Age	Sex	District	Class	Reaction	Avg. BI	BI of SSS
1	31	M	Makwanpur	MB,LL	NR	3.75+	3+
2	25	M	Jhapa	MB,LL	NR	3.5+	4+
3	36	M	Sindhuli	MB,LL	Type 2	4+	4+
4	55	M	Okhaldhunga	MB,LL	Type 1	4+	4+
5	66	M	Kathmandu	MB,LL	NR	4.25+	4+
6	35	M	Sindhupalchok	MB,LL	NR	4.25+	5+
7	51	F	Dolakha	MB,LL	Type 1	4+	5+
8	30	F	Sindhupalchok	MB,BL	Type 2	4.25+	4+
9	50	F	Bhaktapur	MB,LL	Type 2	4+	4+
10	21	M	Shankhuwasabha	MB,LL	Type 2	3.75+	4+
11	35	F	Dhading	MB,LL	Type 1	5.5+	5+
12	10	M	Bara	MB,LL	NR	1.5+	4+
13	35	M	Bara	MB,LL	Type 2	3.75+	4+
14	29	M	Bara	MB,LL	NR	3.25+	4+
15	38	M	Bara	MB,BL	NR	2.25+	5+
16	38	F	Nuwakot	MB,LL	NR	4+	4+
17	35	M	Rautahat	MB,LL	Type 2	5+	6+
18	55	M	Bajura	MB,BL	NR	2+	4+
19	34	F	Lalitpur	MB,BL	NR	2+	4+
20	42	F	Lalitpur	MB,LL	Type 2	4.5+	5+
21	22	F	Kavre	MB,LL	Type 2	1+	4+
22	24	M	Sarlahi	MB,LL	Type 2	3.75+	4+
23	30	F	Saptari	MB,LL	Type 2	2.25+	3+
24	36	M	India	MB,LL	NR	3+	3+
25	65	F	Nuwakot	MB,LL	n/a	4+	4+
26	41	M	Saptari	MB,LL	NR	4+	4+
27	24	M	Baitadi	MB,LL	Type 2	4+	4+
28	40	F	Lalitpur	MB,BB	Type 1	4+	4+
29	57	M	Syangja	MB,LL	NR	3.75+	3+
30	13	M	Gorkha	MB,LL	Type 2	4.25+	4+
31	29	M	Terathum	MB,LL	Type 1	2.5+	4+
32	40	F	Dhading	MB,BL	Type 2	4.25+	5+
33	25	F	n/a	MB,BL	n/a	4+	4+
34	54	M	Syangja	MB,BL	Type 1	1.75+	4+
35	36	M	Dhading	MB,LL	NR	4+	4+
36	28	M	Nuwakot	MB,LL	Type 2	4+	5+
37	25	M	Nuwakot	MB,BL	Type 1	2.5+	3+
38	23	F	Nuwakot	MB,LL	Type 2	3.5+	4+
39	43	M	Nuwakot	MB,LL	Type 2	3.75+	3+
40	32	M	Kathmandu	MB,LL	Type 2	4.25+	5+

Based on the patient information, samples were tabulated according to their region of residence within Nepal. Patients from the Eastern Development Region (EDR), Western Development Region (WDR), Far Western Development Region (FWDR) and Central Development Region (CDR) were recruited in this study (Table 9). No representative sample from Mid-Western Development Region was present probably due to absence of any patient from that locality or the patient was not an MB patient, if present. Information regarding 39 pateints could be retrieved with one patient belonging to India. Information about locality of residence of one of the patients could not be retrieved (Table 5.2). Majority of the patients in this study were from CDR accounting for 67% while 15% were from EDR.

Table 9 : Sample Geographic Distribution by districts and development region. 40 SSS samples were collected from Nepalese patients attending Anandaban Hospital. Patients came from 4 of the 5 development regions of Nepal: EDR = Eastern Development Region; CDR = Central Development Region; WDR = Western Development Region; FWDR = Far-Western Development Region.

District	n	District	N
Jhapa	1	Bara	4
Okhaldhunga	1	Bhaktapur	1
Saptari	2	Dhading	3
Shankhuwasabha	1	Dolakha	1
Terathum	1	Kathmandu	2
EDR	**6**	Kavrepalanchok	1
		Lalitpur	3
Gorkha	1	Makwanpur	1
Syangja	2	Nuwakot	6
WDR	**3**	Rautahat	1
		Sarlahi	1
Baitadi	1	Sindhuli	1
Bajura	1	Sindhulpalchok	2
FWDR	**2**	**CDR**	**27**



* 1 patient was from India; Data on one sample missing.

Similary, the information on 40 samples were analyzed to classify the study population acccording to gender. Females represented 35% of the study sample and males represented 65% of the study sample (Fig. 3).

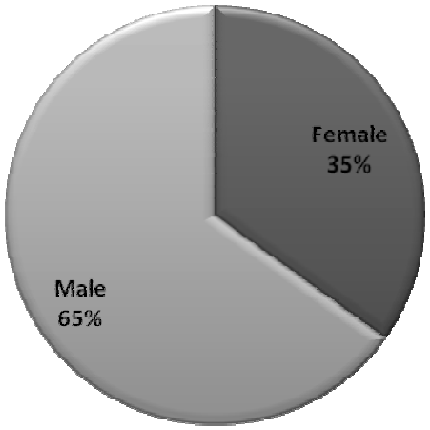


Fig. 3 Gender distribution of the study samples. Of the 40 samples, 26 (65%) were male and 14 (35%) were female.

Based on age, three classes were identified (Fig. 4). Patients with age less than 15 years were classified as Children, those from 15yrs-49yrs were classified as Young people and adults and those over 49yrs were classified as Aged people (Barker and Hall, 1991). Based on age, 40 study samples were distributed as following:

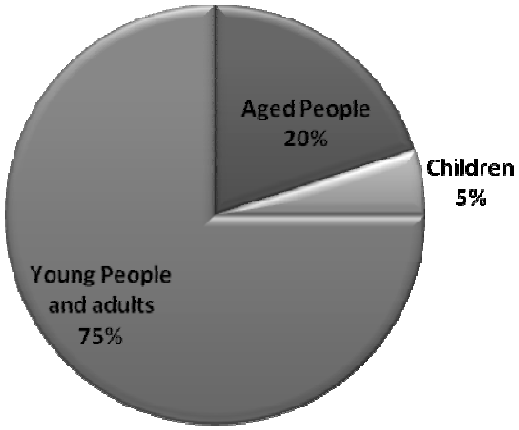


Fig. 4 Age distribution of Nepalese study samples.

5.2 Reactionary state of the patients

While some patients never evidenced reaction, some patients had reaction at the time of diagnosis or later developed a reaction during the time course of this study (Fig 5). 14 (35%) patients experienced no reaction, 7 (17%) patients experienced Type 1 reaction and 17(43%) patients experienced Type 2 reaction at some stage. No conversions from one reactional type to another were observed. Data on two patients (5%) are missing.

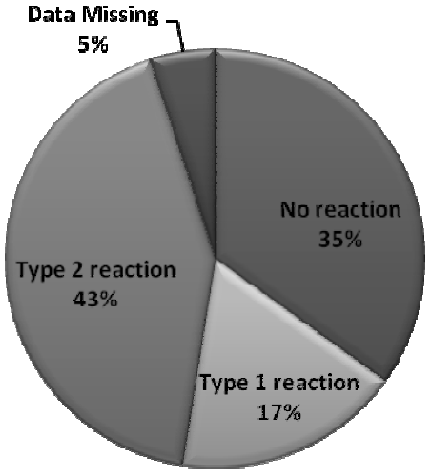


Fig. 5 Reactions within the study population. Reaction status of patients was noted at diagnosis or during the study period.

5.3 DNA extraction from SSS samples

SSS samples from 168 patients was subjected to DNA extraction. Only 40 SSS samples were processed for further analysis. The selection of the 40 samples was based on high BI or good quality of DNA as indicated by measured $A_{260/280}$ values. The yield and purity of the extracted DNA are depicted in Table 10. The yield of the DNA from SSS ranged from 10.1 μg/ml to 265.5 μg/ml for the 40 samples. Similarly, the purity of DNA preparation as indicated by $A_{260/280}$ value ranged from 0.83 to 1.45 with an average value of 1.023.

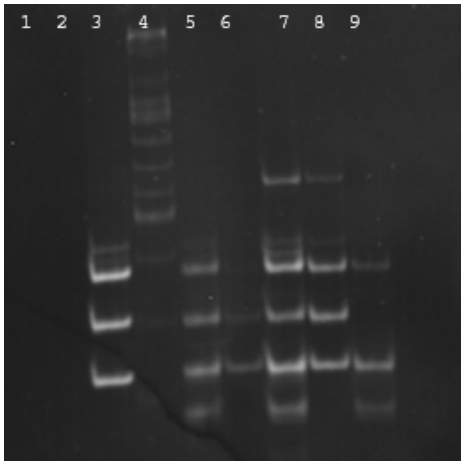
Table 10: Yield and purity of DNA extracted from SSS samples. Concentration of DNA extracted from 40 of the 168 SSS samples measured in μg/ml and purity of the DNA preparation as indicated by $A_{260/280}$ values; S.No. = Sample Number; BI = Bacillary Index; Conc. = Concentration

S.No.	BI	Conc. Of DNA (μg/ml)	$A_{260/280}$
1	3+	128.3	0.93
2	4+	164	1
3	4+	78.5	1.05
4	4+	240.4	0.99
5	4+	155.8	1.02
6	5+	98.2	1.45
7	5+	135.6	0.91
8	4+	62.5	0.83
9	4+	127.1	0.9
10	4+	126.8	0.84
11	5+	90	0.84
12	4+	10.1	1.01
13	4+	72.8	1.18
14	4+	156.3	0.96
15	5+	134.4	1.06
16	4+	134.9	0.97
17	6+	201.3	1.05
18	4+	237.6	1.03
19	4+	172.2	1.08
20	5+	131.6	1
21	4+	123	0.99
22	4+	95.6	1.11
23	3+	142.2	1.01
24	3+	73.2	0.95
25	4+	101.2	1.05
26	4+	265.5	1.06
27	4+	190.7	1.11
28	4+	93.6	0.94
29	3+	194.8	1.1
30	4+	193.8	1.13
31	4+	160.7	1.04
32	5+	142.4	1.09
33	4+	78.1	0.99
34	4+	178.7	0.97
35	4+	217	0.93
36	5+	96.1	0.94
37	3+	129.1	1.09
38	4+	86	0.99
39	3+	87.7	1.16
40	5+	71.5	1.17

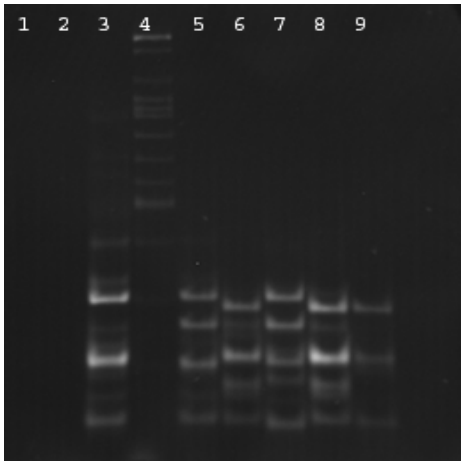
5.4 PCR amplification and DNA fragment analysis of 18 VNTR loci

Multiplex PCR amplification was performed for all 40 SSS samples in four VNTR primer combinations. Combinations 1 and 2 contained primer pairs for 5 VNTR loci while combinations 3 and 4 contained primer pairs for 4 VNTR loci as indicated in Table 7. Resulting amplicons were resolved in 8% polyacrylamide gel for preliminary identification (Fig. 6) and then analyzed by DNA fragment analysis (Fig. 7, 8 and 9). The electropherograms were analyzed to determine the amplicon sizes of each peak and compared against standard NHDP63 strain (Fig. 7) to calculate the repeat copy numbers at each VNTR loci.

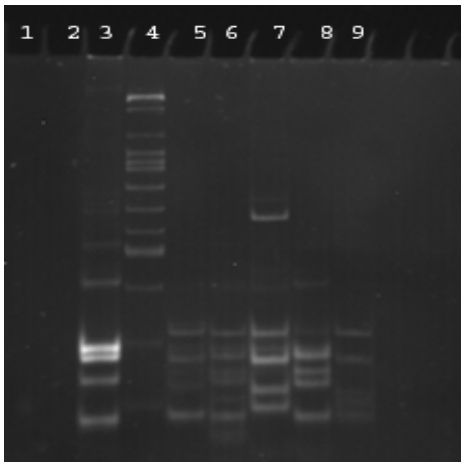
Combination 1



Combination 2



Combination 3



Combination 4

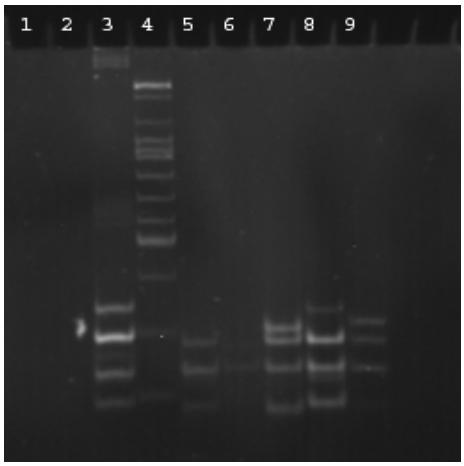


Fig 6. Resolution of Multiplex-PCR amplification product of VNTR loci within Comb.1 through Comb.4 in 8% polyacrylamide gel. Samples: Negative control (lane 2), NHDP63 (lane 3), 100bp Marker (lane 4), 3d/B (lane 5), 24b (lane 6), 26d/B (lane 7), 135c (lane 8) and 36d/B (lane 9)

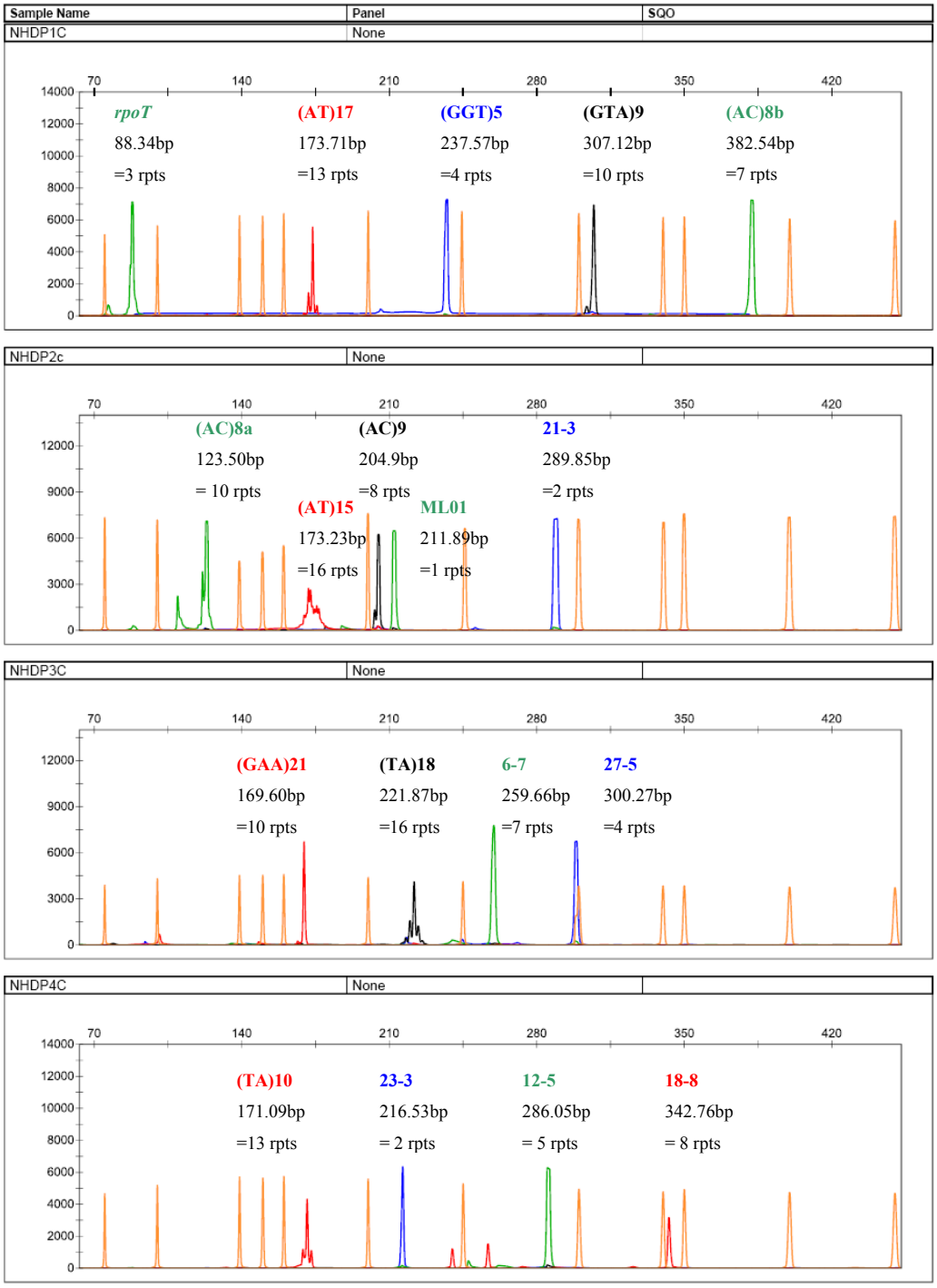


Fig 7 MLVA analysis of standard NHDP63 strain. Each panel represents the multiplex electrophoresis results for Comb.1 through Comb.4 compared against the LIZ-500 DNA standard (orange peaks) on the ABI310 Genetic Analyzer.

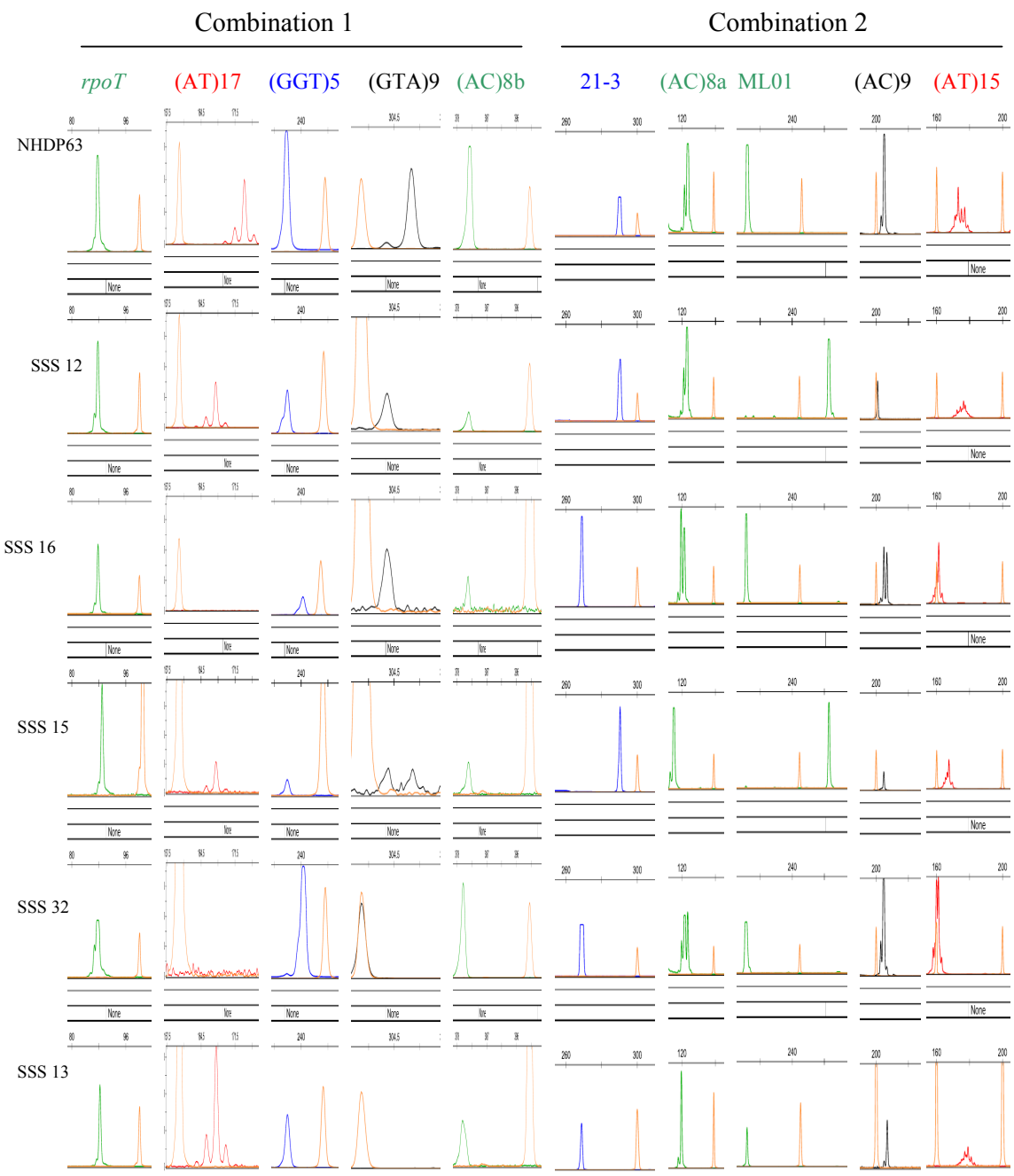


Fig 8. Fragment length analysis of 5 VNTR loci of Combination 1 and 2 in 5 of the *M. leprae* strains from slit skin scrapings of Nepali leprosy patients. Comb#1 includes *rpoT*, (AT)17, (GGT)5, (GTA)9 and (AC)8b. Comb#2 includes 21-3, (AC)8a, ML01, (AC)9 and (AT)15. NHDP63 is positive control and 3d/B, 24b, 26d/B, 135c, 36d/B are clinical samples. Orange peaks indicate internal DNA standard. Peaks of other color indicate indicated loci.

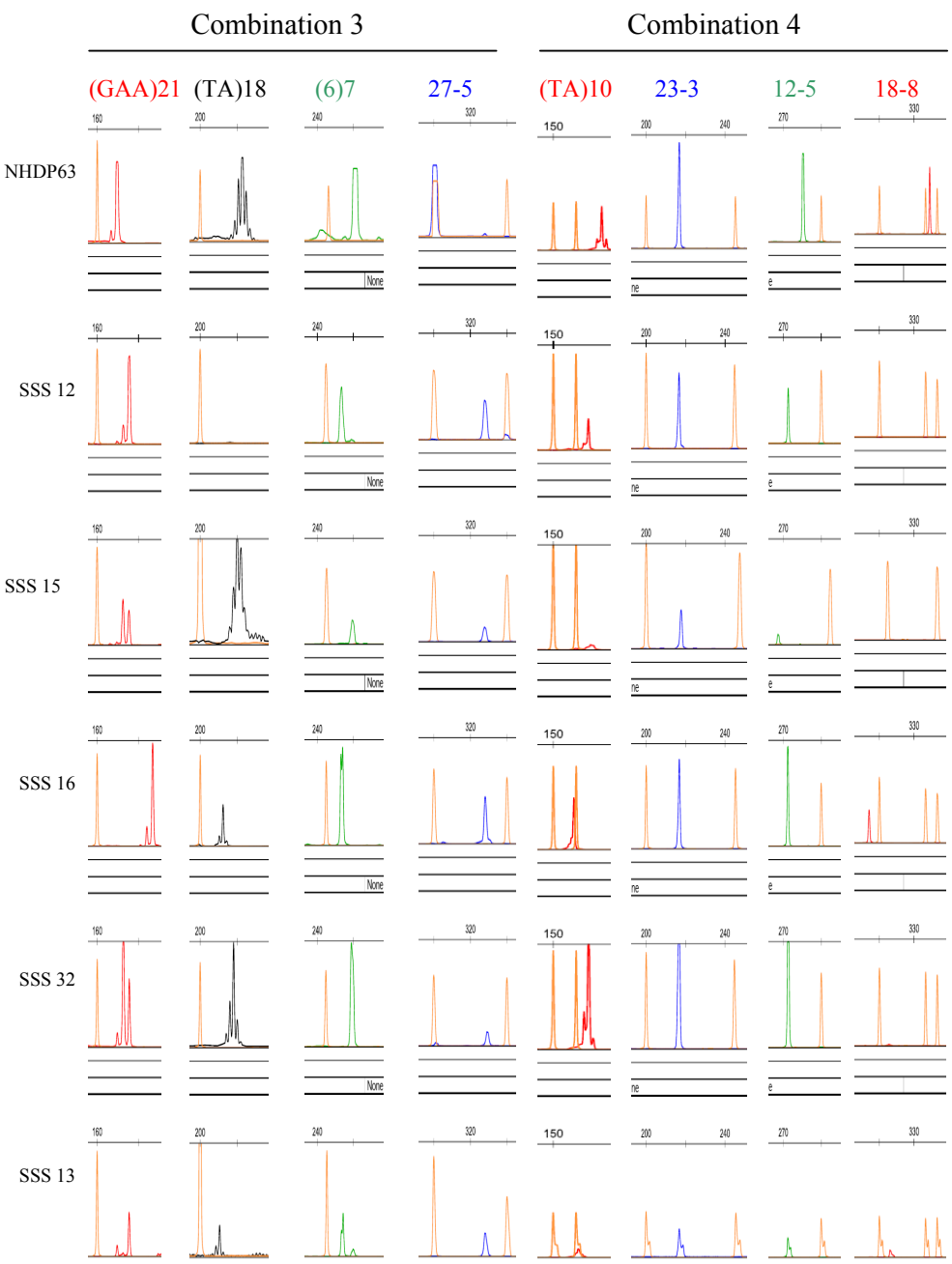


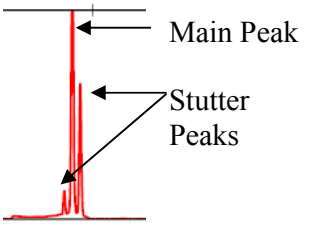
Fig 9. Fragment length analysis of 4 VNTR loci of Combination 3 and 4 in 5 of the *M. leprae* strains from slit skin scrapings of Nepali leprosy patients. Comb#3 includes (GAA)21, (TA)18, 6-7 and 27-5. Comb#4 include (TA)10, 23-3, 12-5 and 18-8. NHDP63 is positive control and 3d/B, 24b, 26d/B, 135c, 36d/B are clinical samples. Orange peaks indicate internal DNA standard. Peaks of other color indicate indicated loci.

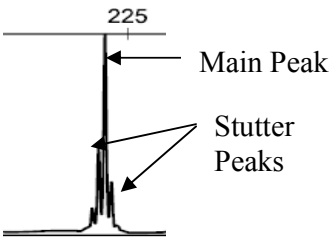
5.5 Stutter Phenomenon

When the electropherograms were analyzed, multiple additional peaks at expected VNTR loci could be seen. These peaks are known as stutter peaks and could be identified as smaller minor peaks occurring as multiples of repeat copy numbers when compared with the major allele peak. Stutter peaks were highly common with locus (TA)18 and (AT)15. Table 11 presents stutter phenomenon seen over VNTR loci in *M. leprae*. Stutter peaks at corresponding loci can be seen in Figure 5.6 and 5.7. It was observed that minisatellite loci were less prone to stutter phenomenon while microsatellite loci were comparably more prone to it.

Table 11: Stutter phenomenon in the VNTR loci. A specific pattern of stutter peak appearance was characteristic for each loci. +1 through +4 indicate products with higher multiple repeat units and -1 through -4 indicate those with lesser multiple repeat units. Stutter products when present are indicated by "+" sign at corresponding loci. The figure to the right shows the distinction between main peak and stutter peaks.

Loci	Stutter Products							
	-4	-3	-2	-1	+1	+2	+3	+4
Comb.1								
(AC)8b	-	-	-	-	-	-	-	-
(GTA)9	-	-	-	+	+	-	-	-
(GGT)5	-	-	-	-	-	-	-	-
(AT)17	-	-	+	+	+	-	-	-
rpoT	-	-	-	-	-	-	-	-
Comb.2								
21-3	-	-	-	-	-	-	-	-
(AC)9	-	-	-	+	-	-	-	-
(AT)15	-	-	+	+	+	+	+	-
(AC)8a	-	-	-	+	-	-	-	-
ML01	-	-	-	-	-	-	-	-
Comb.3								
27-5	-	+	+	+	-	-	-	-
6-7	-	-	-	+	-	-	-	-
(TA)18	+	+	+	+	+	+	+	-
(GAA)21	-	-	-	+	+	-	-	-
Comb.4								
18-8	-	-	-	-	-	-	-	-
12-5	-	-	-	-	-	-	-	-
23-3	-	-	-	-	-	-	-	-
(TA)10	-	-	+	+	+	+	-	-





5.6 Allelic profile of *M. leprae* strains

Of the 18 VNTR loci, 8 loci were minisatellite and 10 loci were microsatellite. All 40 strains did not show any variation at the *rpoT* loci with a copy number 3 at this loci. Loci 23-3, (AC)8b, (GGT)5 were considerably less polymorphic exhibiting only two alleles among the study sample. Similarly, loci 21-3, ML01, 27-5, 12-5, 6-7 had only three alleles among the isolated strains. Overall, microsatellites were more highly polymorphic where multiple alleles could be detected and minisatellites were considerably more stable (Table 12 through 15). In the majority of the samples, 18-8 locus failed to amplify and hence was excluded from further analysis.

The data was further analyzed by Microsatellite toolkit (MS-toolkit) to calculate diversity at each of the seventeen loci (Table 16) and also to calculate diversity index (h) for each loci (Table 17). Calculated values less than 0.3 for the diversity index (h) was considered less discriminatory. (AC)8b, 23-3 and 12-5 were found to be less discriminatory by this classification. Locus *rpoT* had h = 0 and was the non-discriminatory loci. (GGT)5, 21-3, ML01 and 6-7 had values for h ranging from 0.3 to 0.6 and were considered moderately discriminatory. (AC)8a, (GTA)9, (TA)10, (AC)9, (GAA)21, (AT)17, (AT)15, (TA)18 had values for h greater than 0.6 and were considered highly discriminatory (Table 16). 18-8 locus was exempted from analysis due to insufficient data.

Table 12: Allelic profile of *M. leprae* isolates from slit-skin scrapings for Combination 1. Amplicons' sizes were determined in bp and were compared with known NHDP63 strain to determine the corresponding number of repeat units. Combination 1 included *rpoT*, (GGT)5, (AT)17, (GTA)9 and (AC)8b loci; bp = base pairs as determined by FLA; n = allele no.; ND = Not detected

	Combination 1									
Loci	***rpoT***		**(GGT)5**		**(AT)17**		**(GTA)9**		**(AC)8b**	
SSS ID	**bp**	**n**	**bp**	**n**	**bp**	**n**	**Bp**	**n**	**bp**	**n**
NHDP63	**88.34**	**3**	**237.57**	**4**	**173.71**	**13**	**307.12**	**10**	**382.54**	**7**
1	88.03	**3**	240.67	**5**	169.55	**11**	300	**8**	381.66	**7**
2	87.9	**3**	237.42	**4**	169.43	**11**	300.13	**8**	382.02	**7**
3	88.53	**3**	237.51	**4**	169.59	**11**	303.57	**9**	381.78	**7**
4	88.46	**3**	240.64	**5**	171.62	**12**	303.7	**9**	381.24	**7**
5	87.89	**3**	237.31	**4**	165.48	**9**	310.46	**11**	381.82	**7**
6	87.8	**3**	237.07	**4**	ND	**ND**	310.36	**11**	382.59	**7**
7	88.04	**3**	237.39	**4**	169.57	**11**	303.59	**9**	381.79	**7**
8	87.21	**3**	237.6	**4**	ND	**ND**	ND	**ND**	379.91	**6**
9	87.68	**3**	241.31	**4**	ND	**ND**	313.44	**12**	381.36	**7**
10	87.81	**3**	236.49	**4**	177.5	**15**	ND	**ND**	381.35	**7**
11	87.88	**3**	237.19	**4**	171.21	**12**	ND	**ND**	ND	**ND**
12	87.72	**3**	237.48	**4**	167.57	**10**	303.47	**9**	381.49	**7**
13	87.76	**3**	237.35	**4**	167.56	**10**	ND	**ND**	379.66	**6**
14	88.87	**3**	237.42	**4**	167.49	**10**	303.58	**9**	381.55	**7**
15	88.98	**3**	237.39	**4**	167.55	**10**	303.76	**9**	381.45	**7**
16	87.5	**3**	240.38	**5**	ND	**ND**	303.34	**9**	381.37	**7**
17	87.81	**3**	236.91	**4**	169.13	**11**	310.31	**11**	382.1	**7**
18	87.98	**3**	240.63	**5**	171.33	**12**	303.79	**9**	383.1	**7**
19	87.91	**3**	237.43	**4**	165.42	**9**	303.65	**9**	382.71	**7**
20	87.86	**3**	237.25	**4**	179.17	**16**	290.83	**4**	382.57	**7**
21	87.75	**3**	240.21	**5**	173.36	**13**	300	**8**	382.52	**7**
22	87.76	**3**	236.9	**4**	ND	**ND**	ND	**ND**	ND	**ND**
23	88.04	**3**	236.39	**4**	167.44	**10**	ND	**ND**	382.39	**7**
24	87.64	**3**	237.05	**4**	169.17	**11**	313.67	**12**	382.77	**7**
25	87.55	**3**	237.24	**4**	ND	**ND**	310.59	**11**	381.08	**7**
26	87.96	**3**	238	**4**	167.72	**10**	310.88	**11**	381.73	**7**
27	87.75	**3**	240.27	**5**	ND	**ND**	ND	**ND**	383.02	**7**
28	88.62	**3**	240.6	**5**	175.5	**14**	300	**8**	379.89	**6**
29	87.8	**3**	240.28	**5**	ND	**ND**	307.24	**10**	381.37	**7**
30	88.5	**3**	240.47	**5**	171.61	**12**	303.4	**9**	380.71	**7**
31	87.87	**3**	237.48	**4**	169.29	**11**	310.32	**11**	382.33	**7**
32	88.03	**3**	240.2	**5**	173.22	**13**	300	**8**	379.84	**6**
33	87.69	**3**	236.6	**4**	169.05	**11**	303.52	**9**	381.8	**7**
34	86.66	**3**	236.84	**4**	173.31	**13**	ND	**ND**	380.3	**7**
35	87.68	**3**	237.32	**4**	173.58	**13**	293.4	**5**	381.79	**7**
36	88.42	**3**	240.59	**5**	171.64	**12**	300	**8**	381.53	**7**
37	87.56	**3**	237.44	**4**	167.39	**10**	ND	**ND**	ND	**ND**
38	87.6	**3**	237.28	**4**	ND	**ND**	303.6	**9**	381.35	**7**
39	88.28	**3**	237.49	**4**	176.61	**14**	303.68	**9**	381.08	**7**
40	89.14	**3**	237.67	**4**	169.64	**11**	307.14	**10**	381.36	**7**

Table 13: Allelic profile of *M. leprae* isolates from slit-skin scrapings for Combination 2. Amplicons' sizes were determined in bp and were compared with known NHDP63 strain to determine the corresponding number of repeat units. Combination 2 included (AC)8a, 21-3, (AT)15, (AC)9 and ML01 loci; bp = base pairs as determined by FLA; n = allele no.; ND = Not detected

	Combination 2									
Loci	**(AC)8a**		**21-3**		**(AT)15**		**(AC)9**		**ML01**	
SSS ID	**bp**	**n**	**bp**	**n**	**bp**	**n**	**bp**	**n**	**bp**	**n**
NHDP63	**123.5**	**10**	**289.9**	**2**	**173.2**	**16**	**204.9**	**8**	**211.9**	**1**
1	121.5	**9**	ND	**ND**	ND	**ND**	ND	**ND**	ND	**ND**
2	121.7	**9**	290.1	**2**	169.3	**14**	206.8	**9**	263.3	**2**
3	119.8	**8**	290.7	**2**	171.5	**15**	201.2	**6**	263.3	**2**
4	121.2	**9**	ND	**ND**	161.5	**10**	204.8	**8**	211.1	**1**
5	119.7	**8**	269.2	**1**	169.4	**14**	206.7	**9**	262.9	**2**
6	122	**9**	290.7	**2**	171.4	**15**	202.9	**7**	263.2	**2**
7	119.9	**8**	290.8	**2**	175.5	**17**	210.6	**11**	263.3	**2**
8	119.5	**8**	289.8	**2**	163.3	**11**	204.9	**8**	211.6	**1**
9	123.7	**10**	313.5	**3**	167.3	**13**	208.9	**10**	264.1	**2**
10	124.5	**9**	289.2	**2**	167.1	**13**	ND	**ND**	263.6	**2**
11	121.5	**9**	289.7	**2**	162.7	**10**	ND	**ND**	263.5	**2**
12	123.3	**10**	290.5	**2**	177.2	**18**	201.2	**6**	263.3	**2**
13	119.7	**8**	268.7	**1**	179.1	**19**	206.9	**9**	211.8	**1**
14	119.7	**8**	289.9	**2**	173	**16**	201.2	**6**	263.5	**2**
15	119.4	**8**	290.4	**2**	167.4	**13**	204.8	**8**	263	**2**
16	119.5	**8**	269.2	**1**	161.3	**10**	204.4	**8**	211.6	**1**
17	120	**8**	289.8	**2**	169.9	**14**	201.5	**6**	264.7	**2**
18	129.4	**13**	268.9	**1**	160	**9**	205.2	**8**	213	**1**
19	123.8	**10**	290	**2**	171.7	**15**	201.6	**6**	264.8	**2**
20	121.9	**9**	289.7	**2**	171.7	**15**	207.1	**9**	264.6	**2**
21	123.7	**10**	268.4	**1**	160.9	**10**	205	**8**	212.7	**1**
22	124	**10**	ND	**ND**	ND	**ND**	ND	**ND**	315	**3**
23	121.6	**9**	289.4	**2**	172.9	**16**	ND	**ND**	263.8	**2**
24	ND	**ND**	ND	**ND**	ND	**ND**	ND	**ND**	ND	**ND**
25	117.8	**7**	290.2	**2**	163.3	**11**	210.5	**11**	263	**2**
26	121.9	**9**	290.5	**2**	167.2	**13**	201.2	**6**	263.9	**2**
27	121.4	**9**	268.9	**1**	161.2	**10**	206.8	**9**	211.6	**1**
28	121.8	**9**	269.2	**1**	165.5	**12**	202.9	**7**	211.2	**1**
29	123.5	**10**	269.4	**1**	161.5	**10**	204.8	**8**	211.5	**1**
30	121.6	**9**	269.2	**1**	161.5	**10**	206.6	**9**	211.5	**1**
31	121.7	**9**	290.9	**2**	169.2	**14**	203.1	**7**	263.5	**2**
32	122	**9**	269.7	**1**	161.1	**10**	205	**8**	211.7	**1**
33	119.7	**8**	289.2	**2**	171.6	**15**	204.9	**8**	263.9	**2**
34	117.7	**7**	289	**2**	165.7	**12**	ND	**ND**	264	**2**
35	119.9	**8**	290.2	**2**	179.2	**19**	201.2	**6**	263.3	**2**
36	125.5	**11**	269	**1**	161.3	**10**	204.9	**8**	211.6	**1**
37	121.7	**9**	290.5	**2**	176.9	**18**	203.1	**7**	263.5	**2**
38	123.8	**10**	290.2	**2**	171.2	**15**	200.7	**6**	263.4	**2**
39	123.2	**10**	290.5	**2**	171.4	**15**	201	**6**	262.9	**2**
40	121.5	**9**	290.5	**2**	179.2	**19**	201.1	**6**	263.1	**2**

Table 14: Allelic profile of *M. leprae* isolates from slit-skin scrapings for Combination 3. Amplicons' sizes were determined in bp and were compared with known NHDP63 strain to find out corresponding number of repeat units. Combination 3 included 27-5, 6-7, (TA)18 and (GAA)21 loci;; bp = base pairs as determined by FLA; n = allele no.; ND = Not detected

	Combination 3							
Loci	**27-5**		**6-7**		**(TA)18**		**(GAA)21**	
SSS ID	**bp**	**n**	**bp**	**n**	**bp**	**n**	**bp**	**n**
NHDP63	**300.27**	**4**	**259.66**	**7**	**221.87**	**16**	**169.6**	**10**
1	ND	**ND**	253.47	**6**	ND	**ND**	175.28	**12**
2	327.98	**5**	247.4	**5**	212.32	**11**	183.95	**15**
3	328.1	**5**	259.17	**7**	220.06	**15**	175.47	**12**
4	327.93	**5**	259.23	**7**	ND	**ND**	181	**14**
5	328.12	**5**	259.28	**7**	212.28	**11**	175.4	**12**
6	ND	**ND**	247.38	**5**	229.91	**20**	178.47	**13**
7	ND	**ND**	253.39	**6**	210.28	**10**	186.95	**16**
8	ND	**ND**	259.25	**7**	ND	**ND**	186.85	**15**
9	328.16	**5**	259.78	**7**	212.25	**11**	173.07	**11**
10	328	**5**	259.35	**7**	251.37	**31**	186.73	**16**
11	327.85	**5**	253.36	**6**	ND	**ND**	189.61	**17**
12	328.11	**5**	253.42	**6**	ND	**ND**	176	**12**
13	328.07	**5**	254.39	**6**	210.49	**10**	175.45	**12**
14	274.29	**3**	253.48	**6**	218.22	**14**	206.36	**23**
15	328.06	**5**	253.15	**6**	212.35	**11**	186.83	**16**
16	327.92	**5**	259.59	**7**	219.96	**15**	172.49	**11**
17	327.67	**5**	259.24	**7**	215.76	**13**	186.79	**16**
18	327.58	**5**	259.37	**7**	219.71	**15**	183.93	**15**
19	327.76	**5**	253.31	**6**	219.65	**15**	175.59	**12**
20	327.79	**5**	253.13	**6**	223.62	**17**	181.24	**14**
21	237.79	**5**	259.07	**7**	217.73	**14**	172.79	**11**
22	300.98	**4**	259.39	**7**	ND	**ND**	175.35	**12**
23	ND	**ND**	259.51	**7**	ND	**ND**	175.27	**12**
24	273.85	**3**	259.44	**7**	229.67	**20**	175.27	**12**
25	274.1	**3**	247.23	**5**	227.9	**19**	178.34	**13**
26	327.89	**5**	260	**7**	230.03	**20**	180.78	**14**
27	274.28	**3**	254.3	**6**	241.38	**26**	175.36	**12**
28	ND	**ND**	ND	**ND**	ND	**ND**	175.39	**12**
29	327.91	**5**	253.48	**6**	224.01	**17**	181.13	**14**
30	327.91	**5**	259	**7**	221.89	**16**	172.72	**11**
31	274.27	**3**	253.7	**6**	241.24	**26**	181.22	**14**
32	329	**5**	258.94	**7**	218.03	**14**	172.83	**11**
33	273.85	**3**	259.51	**7**	227.81	**19**	175.35	**12**
34	ND	**ND**	253.33	**6**	ND	**ND**	183.93	**15**
35	ND	**ND**	253.28	**6**	ND	**ND**	178.19	**13**
36	328.29	**5**	260.35	**7**	226	**18**	175.73	**12**
37	327.94	**5**	259.27	**7**	241.38	**26**	175.39	**12**
38	ND	**ND**	ND	**ND**	ND	**ND**	175.5	**12**
39	ND	**ND**	ND	**ND**	ND	**ND**	175.49	**12**
40	ND	**ND**	253	**6**	216	**13**	178.39	**13**

Table 15: Allelic profile of *M. leprae* isolates from slit-skin scrapings for Combination 4. Amplicon size was determined in bp and compared with known NHDP63 strain to find out corresponding number of repeat units. Combination 4 included 23-3, 12-5, (TA)10 and 18-8 loci; bp = base pairs as determined by FLA; n = allele no.; ND = Not detected

	Combination 4							
Loci	**23-3**		**12-5**		**(TA)10**		**18-8**	
SSS ID	**bp**	**n**	**bp**	**n**	**bp**	**n**	**bp**	**N**
NHDP63	**216.5**	**2**	**286.1**	**5**	**171.1**	**13**	**342.8**	**8**
1	ND	**ND**	ND	**ND**	ND	**ND**	ND	**ND**
2	216.7	**2**	273.6	**4**	163.1	**9**	327	**7**
3	216.7	**2**	262.2	**3**	165.4	**10**	ND	**ND**
4	216.7	**2**	273.9	**4**	165.4	**10**	ND	**ND**
5	194.3	**1**	273.8	**4**	161.4	**8**	ND	**ND**
6	216.7	**2**	274.1	**4**	163.4	**9**	ND	**ND**
7	216.7	**2**	273.6	**4**	163.1	**9**	ND	**ND**
8	ND	**ND**	285	**5**	ND	**ND**	ND	**ND**
9	216.8	**2**	261.7	**3**	161.2	**8**	ND	**ND**
10	216.9	**2**	261.7	**3**	165	**10**	ND	**ND**
11	216.9	**2**	273.8	**4**	163.9	**9**	ND	**ND**
12	216.7	**2**	273.8	**4**	165.4	**10**	ND	**ND**
13	216.8	**2**	273.8	**4**	161	**8**	309.5	**6**
14	216.8	**2**	273.5	**4**	165.2	**10**	ND	**ND**
15	216.8	**2**	273.6	**4**	159.1	**7**	291.2	**5**
16	217.8	**2**	266	**3**	167.5	**11**	ND	**ND**
17	216.3	**2**	273.9	**4**	167	**11**	ND	**ND**
18	216.5	**2**	273.7	**4**	169	**12**	ND	**ND**
19	216.4	**2**	273.6	**4**	165	**10**	ND	**ND**
20	216.4	**2**	273.5	**4**	171.2	**13**	ND	**ND**
21	216.2	**2**	273.4	**4**	167	**11**	ND	**ND**
22	ND	**ND**	ND	**ND**	ND	**ND**	ND	**ND**
23	216.4	**2**	273.5	**4**	ND	**ND**	ND	**ND**
24	216.3	**2**	ND	**ND**	161.1	**8**	ND	**ND**
25	216.7	**2**	273.8	**4**	161.3	**8**	309	**6**
26	217	**2**	274.1	**4**	165.2	**10**	309/344	**6/8**
27	216.6	**2**	261.8	**3**	167.3	**11**	ND	**ND**
28	216.8	**2**	273.6	**4**	165.1	**10**	343	**8**
29	216.7	**2**	273.9	**4**	161.5	**8**	ND	**ND**
30	216.7	**2**	274	**4**	165.4	**10**	327	**7**
31	216.9	**2**	273.6	**4**	163.2	**9**	327.1	**7**
32	217	**2**	274.4	**4**	165.6	**10**	309	**6**
33	216.3	**2**	273.6	**4**	165	**10**	ND	**ND**
34	216.3	**2**	273.4	**4**	165.1	**10**	ND	**ND**
35	216.8	**2**	273.8	**4**	163.5	**9**	ND	**ND**
36	217.4	**2**	276.3	**4**	167.5	**11**	ND	**ND**
37	216.3	**2**	273.9	**4**	161.1	**8**	ND	**ND**
38	216.8	**2**	273.6	**4**	165.1	**10**	343.7	**8**
39	216.7	**2**	273.8	**4**	165.5	**10**	343	**8**
40	216.7	**2**	273.6	**4**	167.3	**11**	ND	**ND**

Table 16: Allele diversity for *M. leprae* VNTR loci in Nepal. Distribution of alleles at different VNTR loci and its corresponding frequency of occurrence as assessed by MS-toolkit.; Allele refers to common repeat number found.; n = number of isolates; freq. = frequency of occurrence of the allele. 18-8 loci excluded due to insufficient data

Diversity at indicated VNTR loci											
loci	**Allele**	***n***	**Freq. (%)**	**loci**	**Allele**	***n***	**Freq. (%)**	**loci**	**Allele**	***n***	**Freq. (%)**
rpoT	3	40	100	**23-3**	1	1	2.7	**(AC)8b**	6	4	10.81
21-3	1	11	30.56		2	36	97.3		7	33	89.19
	2	24	66.67	**ML01**	1	12	31.58	**27-5**	3	6	20.69
	3	1	2.78		2	25	65.79		4	1	3.45
6-7	5	3	8.11		3	1	2.63		5	22	75.86
	6	15	40.54	**12-5**	3	5	13.51	**(GGT)5**	4	29	72.5
	7	19	51.35		4	31	83.78		5	11	27.5
(AC)8a	7	2	5.13		5	1	2.7				
	8	11	28.21	**(AT)17**	9	2	6.45	**(GTA)9**	4	1	3.23
	9	16	41.03		10	7	22.58		5	1	3.23
	10	8	20.51		11	9	29.03		8	6	19.35
	11	1	2.56		12	5	16.13		9	13	41.94
	13	1	2.56		13	4	12.9		10	2	6.45
(GAA)21	11	5	12.5		14	2	6.45		11	6	19.35
	12	16	40		15	1	3.23		12	2	6.45
	13	4	10		16	1	3.23	**(TA)18**	10	2	7.14
	14	5	12.5	**(AT)15**	9	1	2.7		11	4	14.29
	15	4	10		10	9	24.32		13	2	7.14
	16	4	10		11	2	5.41		14	3	10.71
	17	1	2.5		12	2	5.41		15	4	14.29
	23	1	2.5		13	4	10.81		16	1	3.57
(AC)9	6	10	30.3		14	4	10.81		17	2	7.14
	7	4	12.12		15	7	18.92		18	1	3.57
	8	10	30.3		16	2	5.41		19	2	7.14
	9	6	18.18		17	1	2.7		20	3	10.71
	10	1	3.03		18	2	5.41		26	3	10.71
	11	2	6.06		19	3	8.11		31	1	3.57
				(TA)10	7	1	2.78				
					8	7	19.44				
					9	6	16.67				
					10	14	38.89				
					11	6	16.67				
					12	1	2.78				
					13	1	2.78				

The results indicated *rpoT* had only 3 repeat units in all the 40 samples studied (Table 16). (TA)18 was the most polymorphic of all when analysed in 40 samples. 12 different alleles were detected at this loci. Similarly, (AT)15 had 11 different alleles and was the

second most polymorphic (Table 16). Interestingly, (AC)8b loci was less polymorphic although being a microsatellite.

Table 17: Diversity indices of *M. leprae* VNTR loci. The discriminatory index (h) was calculated using MS-Toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit). When $h>0.6$, the loci were designated highly discriminatory. When $0.3\leq h\leq 0.6$, the loci were designated moderately discriminatory. When $0<h\leq 0.3$, the loci were designated less discriminatory and when $h=0$, the loci were designated non-discriminatory (Sola *et al.*, 2003).

Loci	Discriminatory Index	Inference
rpoT	0	Non-discriminatory
23-3	0.0541	Less Discriminatory
(AC)8b	0.1982	Less Discriminatory
12-5	0.2868	Less Discriminatory
27-5	0.3941	Moderately Discriminatory
GGT5	0.409	Moderately Discriminatory
21-3	0.4746	Moderately Discriminatory
ML01	0.4794	Moderately Discriminatory
6-7	0.5811	Moderately Discriminatory
(AC)8a	0.7382	Highly Discriminatory
(GTA)9	0.7613	Highly Discriminatory
(TA)10	0.7746	Highly Discriminatory
(AC)9	0.7879	Highly Discriminatory
(GAA)21	0.7974	Highly Discriminatory
(AT)17	0.8387	Highly Discriminatory
(AT)15	0.8859	Highly Discriminatory
(TA)18	0.9339	Highly Discriminatory

*18-8 loci has been excluded due to insufficient data.

5.7 Cluster Analysis of Isolated Strains of M. leprae

Based on the cumulative data on 18 VNTR loci for 40 studied *M. leprae* isolates, strains with similar VNTR patterns were grouped together and assigned to cluster (Table 18). For the cluster analysis, 7 VNTR loci were taken into consideration which had less and moderately discriminatory power as indicated by their diversity index. The loci used for cluster analysis included 7 loci in following order: 23-3, (AC)8b, 12-5, (GGT)5, 21-3, ML01 and 27-5. Four major clusters were identified along with four unique strains which did not share any similarity with other groups (Table 5.10). Cluster A had VNTR signature of 2-7-4-4-2-2-3/5 for the loci considered. Cluster B had VNTR signature of 2-7-3/4-5-1-1-3/5 for the loci considered. Similarly, Cluster C had VNTR signature of 2-7-3-4-2/3-2-5. Cluster D had VNTR signature of 2-6-4-5/4-1-1-5for the loci considered. Further sub-classification was performed when the strains within the major cluster differed at one or two VNTR loci while retaining its cluster signature. 18-8 and (TA)10 loci has not been included in the analysis of cluster because the majority of the samples did not show any detectable product at these loci. Similarly, locus rpoT was invariable among all 40 samples with repeat copy number 3 and is excluded for cluster analysis. The data for excluded loci have not been shown in Table 18.

Table 18: Cluster analysis of 40 *M. leprae* strains. 40 *M. leprae* isolates were arranged based on the similarity in alleles at different loci. Four major clusters (A-D) could be observed sharing similar VNTR patterns when the loci *rpoT*, 23-3, AC8b, 12-5, 27-5, GGT5, 21-3 and ML01 were taken into consideration. Four strains had unique VNTR patterns.

SSS#	23-3	(AC)8b	12-5	GGT5	21-3	ML01	27-5	6-7	(AC)8a	(GTA)9	(TA)10	(AC)9	(GAA)21	(AT)17	(AT)15	
26	2	7	4	4	2	2	5	7	9	11	10	6	14	10	13	Cluster A.1
37	2		4	4	2	2	5	7	9		8	7	12	10	18	
11	2		4	4	2	2	5	6	9		9		17	12	10	
23	2	7	4	4	2	2		7	9				12	10	16	
17	2	7	4	4	2	2	5	7	8	11	11	6	16	11	14	
20	2	7	4	4	2	2	5	6	9	4	13	9	14	16	15	
19	2	7	4	4	2	2	5	6	10	9	10	6	12	9	15	
12	2	7	4	4	2	2	5	6	10	9	10	6	12	10	18	
15	2	7	4	4	2	2	5	6	8	9	7	8	16	10	13	
2	2	7	4	4	2	2	5	5	9	8	9	9	15	11	14	
7	2	7	4	4	2	2		6	8	9	9	11	16	11	17	
34	2	7	4	4	2	2		6	7		10		15	13	12	
35	2	7	4	4	2	2		6	8	5	9	6	13	13	19	
38	2	7	4	4	2	2			10	9	10	6	12		15	
39	2	7	4	4	2	2			10	9	10	6	12	14	15	
40	2	7	4	4	2	2		6	9	10	11	6	13	11	19	
6	2	7	4	4	2	2		5	9	11	9	7	13		15	
24	2	7		4			3	7		12	8		12	11		Cluster A.2
33	2	7	4	4	2	2	3	7	8	9	10	8	12	11	15	
14	2	7	4	4	2	2	3	6	8	9	10	6	23	10	16	
31	2	7	4	4	2	2	3	6	9	11	9	7	14	11	14	
25	2	7	4	4	2	2	3	5	7	11	8	11	13		11	
4	2	7	4	5		1	5	7	9	9	10	8	14	12	10	Cluster B.1
18	2	7	4	5	1	1	5	7	13	9	12	8	15	12	9	
21	2	7	4	5	1	1	5	7	10	8	11	8	11	13	10	
30	2	7	4	5	1	1	5	7	9	9	10	9	11	12	10	
36	2	7	4	5	1	1	5	7	11	8	11	8	12	12	10	
29	2	7	4	5	1	1	5	6	10	10	8	8	14		10	
16	2	7	3	5	1	1	5	7	8	9	11	8	11		10	Cluster B.2
27	2	7	3	5	1	1	3	6	9		11	9	12		10	Cluster B.3
10	2	7	3	4	2	2	5	7	9		10		16	15	13	Cluster C.1
3	2	7	3	4	2	2	5	7	8	9	10	6	12	11	15	
9	2	7	3	4	3	2	5	7	10	12	8	10	11		13	Cluster C.2
32	2	6	4	5	1	1	5	7	9	8	10	8	11	13	10	Cluster D.1
28	2	6	4	5	1	1			9	8	10	7	12	14	12	
13	2	6	4	4	1	1	5	6	8		8	9	12	10	19	Cluster D.2
8		6	5	4	2	1		7	8			8	15		11	Unique Strains
22				4		3	4	7	10				12			
1		7		5				6	9	8			12	11		
5	1	7	4	4	1	2	5	7	8	11	8	9	12	9	14	

Chapter 6
Discussion and Conclusion

6.1 Discussion

New VNTR technologies are currently becoming the global standard for leprosy molecular epidemiology. The purpose of this project was to test the implementation in Nepal of SSS-DNA isolation for VNTR sampling, multiplex processing and FLA analysis. The results from the study indicate that the typing of *M. leprae*, based on VNTR, is possible in Nepal using techniques such as multiplex PCR performed with fluorescent tagged primers and DNA fragment analysis for accurate estimation of the repeat polymorphisms. Moreover, determination of correct repeat copy number at VNTR loci using DNA fragment analysis has also helped to classify *M. leprae* strains circulating within Nepal into different clusters. Classification into such clusters could help to decipher the epidemiological link between the strains.

Geographically, of the 40 selected patients, 67% lived in the Central Development Region (n=27), 15% lived in Eastern Development Region (n=6) and the remaining 18% came from other regions like Western Development Region (n=3), Far-Western Development Region (n=2) and India (n=1). National data on leprosy show greatest number of leprosy patients are reported in the CDR followed by EDR (MoHP, 2009/2010). As of mid-November 2009, of the 1721 MB leprosy patients under treatment in Nepal, there were 551 (32.01%) MB patients registerd in CDR followed by 425 (24.69%) MB cases in EDR (MoHP, 2009). As the study sampling sites were in Kathmandu (CDR), it was expected that the majority of samples would be from that district.

Gender-wise, 65% were male (n=26) and 35% female (n=14). National data as of 2007/08 indicates 31.3% female leprosy patients (MoHP, 2007/2008). Similarly, study conducted by Varkevisser et al. (2009) have found that more men than women leprosy patients were registered in four endemic countries: Indonesia, Nigeria, Nepal and Brazil. This discrepancy in figures could possibly be due to strong traditions, low status of

women, their limited mobility, illiteracy and poor knowledge of leprosy (Varkevisser *et al.*, 2009).

The ages of the studied sample ranged from 10 years to 66 years with a mean age 35.95 years. Three classes were defined as Children (<15yrs), Young people and Adults (15-49yrs) and Aged people (>49yrs) (Barker and Hall, 1991). The group children (n=2) constituted 5%, young people and adult (n=30) constituted 75% and the aged people (n=8) constituted 20%. Proportion of child cases in leprosy is significant as it reflects recent transmission (Kimura *et al.*, 2009). National data has recorded an average of 6.72% of new cases among children (MoHP, 2009/2010)(Annual report 2007/08).

In this study, the reaction states of the sample population were also recorded. 18% of the patients were diagnosed with T1R. In a previous Nepalese study, T1R was found among 28.85% of LL patients and 12.9% of BL patients (Adhikari, 2009). Within the context of Nepal, a study by Roche *et al*. (1991) has reported T1R among 52% of the borderline leprosy patients with frequency of occurrence ranging from one to five episodes. A retrospective study of borderline leprosy patients attending a leprosy referral centre in Nepal showed that 30% developed T1R (Van Brakel *et al.*, 1994). The prevalence rate differs with the study conducted in hospital settings and field settings (Kahawita and Lockwood, 2008). Positive slit skin smear and age has been identified as risk factors for T1R development (Ranque *et al.*, 2007; Roche *et al.*, 1991). Also in the present study, among the seven patients who presented with T1R, four were aged 40 years or over while only one of them were between 20-30 years of age and two of the patients were below 30 years of age.

ENL was found among 48% of the MB patients in this study. A study by Adhikari (2009) has shown 28.85% of LL patients and 12.9% of BL patients developing ENL reaction. Similarly, a retrospective study has shown 30% of patients presenting with ENL reaction at the time of diagnosis while 41% developed ENL in the first year of treatment with MDT (Feuth *et al.*, 2008). The risk factors associated with the development of ENL have been identified as a lepromatous leprosy (LL) and BI>4+ (Becx-Bleumink and Berhe, 1992; Manandhar *et al.*, 1999; Pocaterra *et al.*, 2006). In the

present finding also, 15 of the 17 patients with ENL or Type 2 reaction were LL patients and the majority had an average BI greater than or equal to 3.5.

Unlike similar previous studies conducted in Nepal which required a biopsy sample from a patient, this study has been based on protocol designed to process the material collected from SSS. Collection of slit skin scraping is less invasive than a skin biopsy and poses minimal discomfort to the patient. Amplification of VNTR loci in the *M. leprae* DNA extracted from slit skin scrapings has been performed earlier (Sakamuri *et al.*, 2009b). Successful DNA isolation and further processing was performed from 23.8% of 168 BI positive patients sampled.

In some of the samples, the PCR products failed to be detected or were not amplified. *rpoT*, (GGT)5 and (GAA)21 amplified in all 40 samples. However there were one or more samples where one or more loci did not amplify. A null allele is defined as any allele at a microsatellite locus which consistently fails to amplify by PCR (Dakin and Avise, 2004). Such a phenomenon has been attributed to poor primer annealing due to sequence divergence in one or both of the flanking primers (Dakin and Avise, 2004). It is more intensified when mispairing occurs at the 3'-end of primer with the template DNA since it is the site where the extension begins (Kwok *et al.*, 1990). Similarly, template DNA quality and quantity can also cause any loci to not amplify (Dakin and Avise, 2004). In the present study, null alleles observed for various samples could probably be attributed to quality and quantity of template DNA . A purification protocol after DNA extraction from SSS may reduce such null alleles at VNTR loci.

Overall, it was found that the microsatellite loci were more polymorphic than the minisatellite loci as indicated by higher number of alleles and diversity index. For minisatellites, the diversity index (h) ranged from 0 to 0.58 while that for microsatellite ranged from 0.19 to 0.93. The diversity index results (Table 16) indicate a priority of VNTR loci in consideration for evaluating the relatedness among strains. VNTR loci like (TA)18, (AT)17, (AT)15 etc. with a high diversity index would be too variable to make inferences on strain relatedness. Similarly, locus like *rpoT* with zero diversity index is likely unsuitable as it shows no polymorphism at all. Loci 23-3, (AC)8b, 12-5

were less discriminatory. Loci 27-5, (GGT)5, 21-3, ML01 and 6-7 were moderately discriminatory. Microsatllite loci (AC)8a, (GTA)9, (TA)10, (AC)9, (GAA)21, (AT)17, (AT)15 and (TA)18 were highly discriminatory.

Analysis of the allelic profiles of 40 *M. leprae* isolates in this study has revealed that minisatellite markers are less polymorphic than the microsatellite markers which corroborate the findings of previous studies. The locus *rpoT* (6-3a) was stable within these 40 Nepalese *M. leprae* strains with a copy number of three. Across the globe, *rpoT* has been reported with either three repeats or four repeats only. Strains with copy number 3 are reported in Korean populations while those with copy number 4 are prevalent in Brazilian and Haitian populations. In Japanese populations, both types are prevalent (Matsuoka *et al.*, 2000; Zhang *et al.*, 2005). Copy number 3 for *rpoT* has also been reported in South-Indian populations (Shinde *et al.*, 2009) and Chinese populations (Xing *et al.*, 2009).

The 23-3 locus was found to be the second most stable locus investigated with only 2 alleles and the copy number 2 being predominant. The result from this study supports the general finding of copy number two being prevalent among Asian countries like India (Shinde *et al.*, 2009), China (Xing *et al.*, 2009), Philippines (Kimura *et al.*, 2009) and Thailand (Srisungnam *et al.*, 2009).

Similarly, the 12-5 locus had only three detected alleles and the copy number four was found to be prevalent within this Nepalese sample. The locus seems to be non-polymorphic in Chinese populations (Weng *et al.*, 2006) but strains with copy number four have been identified (Xing *et al.*, 2009). Copy numbers 4 and 5 have been found in Indian isolates (Shinde *et al.*, 2009); 3, 4 and 5 in Thai isolates (Srisungnam *et al.*, 2009); 4 and 5 in Philippine isolates (Kimura *et al.*, 2009).

The 27-5 locus has exhibited less polymorphism with three alleles and copy number 5 being the most prevalent. Locus 21-3 and ML01 were polymorphic to a similar extent as indicated by their diversity indices. In this study, 21-3 and ML01 had three alleles with copy number 2 being the predominant in both. The locus is reported invariable in China

(Weng *et al.*, 2006; Xing *et al.*, 2009) while copy number 1 and 3 could also be found in India, although copy number 2 remained the most dominant (Shinde *et al.*, 2009).

The 6-7 locus is the most polymorphic of minisatellite loci analyzed in this study as indicated by its diversity index. Three alleles could be found at 6-7 loci with copy number 7 being the most common. In China, the copy number for this locus ranged from 6 to 10 (Weng *et al.*, 2007; Xing *et al.*, 2009) while in India it ranged from 6 to 9 and 6 being the most common (Shinde *et al.*, 2009). Similar patterns could be observed in Brazil (Fontes *et al.*, 2009), Thailand (Srisungnam *et al.*, 2009), Philippines (Kimura *et al.*, 2009; Sakamuri *et al.*, 2009b) and Colombia (Cardona-Castro *et al.*, 2009a).

Locus 18-8 was not amplified in most of the samples. However, in samples where it could be detected the copy number was 5, 6, 7 or 8. In a Philippine population, 8 was the most common allele (Kimura *et al.*, 2009). In India, 7, 8 and 9 repeat copy numbers were commonly observed at 18-8 (Shinde *et al.*, 2009). In China, 18-8 locus was evaluated as being non-polymorphic (Weng *et al.*, 2006).

In this study 10 total microsatellite loci were analyzed: (GGT)5, (AT)17, (GTA)9, (AC)8b, (AC)8a, (AT)15, (AC)9, (TA)18, (GAA)21 and (TA)10. In general, microsatellite loci appeared more polymorphic than minisatellite loci within Nepal. However, one of the loci, (AC)8b, was considerably less polymorphic. The diversity index for the locus was calculated to be 0.19. Only two alleles, 6 and 7, were reported at this locus with 7 being the dominant. Comparisons with those from India (Shinde *et al.*, 2009) and China (Xing *et al.*, 2009) also do not reveal such vast polymorphism at this locus.

(GGT)5 was the next less polymorphic microsatellite locus. Two alleles, 4 and 5, were reported for this locus with 4 being most common. This locus was found to be non-polymorphic in China (Weng *et al.*, 2006; Xing *et al.*, 2009) while in Indian isolates alleles 4 and 5 were observed (Shinde *et al.*, 2009). Alleles 4,5 and 6 could be observed in Japan (Zhang *et al.*, 2005), Philippines (Kimura *et al.*, 2009), Thailand (Srisungnam *et al.*, 2009).

The other microsatellite loci, (AC)8a, (GTA)9, (TA)10, (AC)9, (GAA)21, (AT)17, (AT)15, and (TA)18 were found to be considerably polymorphic in Nepal as indicated by their diversity indices which ranged from 0.73 to 0.93. Locus (AC)8a demonstrated 7 alleles with copy number 9 being the most common. The locus has not been previously studied in Nepalese populations. Other microsatellite studied for the first time in Nepal included (TA)10 and (AT)15. (AC)8a was similarly found to be polymorphic in China (Weng *et al.*, 2007) but the alleles were quite different from those of this study. In India, the alleles ranged from 7 to 10 and 13 (Shinde *et al.*, 2009). In Thailand, it ranged from 7 to 11 (Srisungnam *et al.*, 2009).

Similarly, (TA)10 and (AT)15 were also highly polymorphic within Nepal. 7 different alleles could be observed for (TA)10 ranging from copy number 7 to 13. For (AT)15, 11 different alleles could be observed with copy numbers ranging from 9 to 19. (AT)15 stands second as a highly discriminatory locus after (TA)18. For (TA)18, 12 alleles could be observed and the discriminatory index for this locus was 0.93. Similar results could be observed for these loci in China (Xing *et al.*, 2009), India (Shinde et al 2009), Brazil (Fontes *et al.*, 2009), Thailand (Srisungnam *et al.*, 2009), Japan (Zhang *et al.*, 2005).

In this study, 6 alleles were observed for (GTA)9 locus. The locus has been found to be highly variable in China which has reported as much as 24 different alleles (Weng *et al.*, 2007; Xing *et al.*, 2009). However, in India the variation is comparably less extensive (Shinde *et al.*, 2009). In a Japanese population, the alleles have ranged from copy number 8 to 16 (Zhang *et al.*, 2005). In Thailand (Srisungnam *et al.*, 2009) and Brazil (Fontes *et al.*, 2009), copy numbers for the locus have ranged from 8 to 15.

For (AC)9 locus, 6 alleles were observed and the copy numbers ranged from 6 to 11. Copy numbers 6 and 8 was found to be most common in Nepal. In the case of (GAA)21 locus, copy number 12 was found to be common in this study. A previous study conducted in Nepal (Pandey, 2005) found wide variation in copy number among 118 Nepalese isolates. Repeat numbers ranged from 3 to 30 with 13 and 15 being the most common. (GAA)21 was also found to be highly polymorphic in China (Weng *et al.*,

2007), India (Shinde *et al.*, 2009), Thailand (Srisungnam *et al.*, 2009), Brazil (Fontes *et al.*, 2009).

Locus (AT)17 is ranked the third most polymorphic within Nepal with its diversity index 0.83. Eight different alleles were observed and the copy numbers ranged from 9 to 16 with 11 being the most common. In India, copy number for this locus has been reported to range from 8 to 17 and 19 (Shinde *et al.*, 2009; Young *et al.*, 2004). Allelic profile for this locus appears to be different according to the country (Cardona-Castro *et al.*, 2009a; Fontes *et al.*, 2009; Srisungnam *et al.*, 2009; Weng *et al.*, 2007).

Previous analyses of VNTR profiles of *M. leprae* isolates from Asian countries has demonstrated a consistent pattern of 5-4-2 allele at 27-5, 12-5 and 23-3 (Sakamuri *et al.*, 2009a; Xing *et al.*, 2009). Although a small sample size, similar patterns were observed in *M. leprae* isolates in Nepalese samples in this study. An association of allele 4 at (GGT)5 locus with allele 2 at 21-3 locus and allele 5 with allele 1 at corresponding loci has been previously demonstrated within global *M. leprae* isolates (Srisungnam *et al.*, 2009). In this study, the same type of association was observed with allele 4 at (GGT)5 locus with allele 2 at 21-3 locus. However, the association of allele 5 with the allele 3 at the corresponding loci was not observed. Instead, the allele 5 at (GGT)5 was found to be associated with allele 1 at 21-3 locus. Allele 3 at 21-3 locus was relatively uncommon among 40 Nepalese *M. leprae* isolates. It was also observed that the alleles at the ML01 locus were also in association with the alleles at (GGT)5 and 21-3 loci. Consistent allele patterns of 4-2-2 and 5-1-1 at loci (GGT)5, 21-3 and ML01 were highly common. Allele 2 at ML01 was highly common in the isolates with the allele 4 at (GGT)5 locus and allele 2 at 21-3 loci. Similarly, allele 1 at ML01 was highly common among isolates with allele 5 at (GGT)5 locus and allele 1 at 21-3 locus.

Based on the similarity of VNTR patterns exhibited by different strains, four major clusters of strains could be identified. The clusters were defined on the basis of similarity in alleles at loci 23-3, (AC)8b, 12-5, (GGT)5, 21-3, ML01 and 27-5. These loci were taken into consideration for cluster analysis since they have been classified as less discriminatory or moderately discriminatory based on their diversity indices. Too

stable loci like *rpoT* and too variable loci like (TA)18, (AT)15, (AT)17, etc. would not yield useful information on strain relatedness.. Cluster A shared the common VNTR signature of 2-7-4-4-2-2-3/5at these loci, respectively. Depending whether the loci 27-5 exhibited allele 5 or 3, the cluster was subdivided as Cluster A.1 and Cluster A.2. Similarly, Cluster B shared the common VNTR signature of 2-7-3/4-5-1-1-3/5at the corresponding loci, respectively. Cluster B could be similarly sub-grouped as Cluster B.1 having alleles 4 and 5 at the loci 12-5 and 27-5, Cluster B.2 having alleles 3 and 5 at those loci and Cluster B.3 having alleles 3 and 3 at the corresponding loci, respectively. Cluster C strains shared the common VNTR signature of 2-7-3-4-2/3-2-5. Sub-grouping of this cluster was based on whether the locus (GGT)5 contained 2 or 3 as an allele. Cluster D strains had the VNTR signature of 2-6-4-5/4-1-1-5. The cluster was similarly sub-grouped on the basis of different alleles at the locus (GGT)5. Four strains were unique in that their VNTR patterns could not be related with any of the defined clusters. The study indicates that the majority of the *M. leprae* strains (22 out of 40 studied *M. leprae* isolates) belonged to Cluster A. The next common strains of *M. leprae* would belong to Cluster B. However, the observed clusters do not seem to be empirically confined within the particular geographical region of Nepal. Strains in the same cluster can be speculated to have come from diverse regions because the patients' residence has been widely distributed in the same cluster.

In this study, samples 39 and 38 have come from the patients of a same family. When the VNTR profiles were evaluated, both shared exactly the same VNTR signature pattern. Finding consistent patterns among such multi-case family (MCF) members indicate the usefulness of multiple-locus VNTR analysis in tracing sources of infection and transmission chains (Sakamuri *et al.*, 2009b). Similar cases among MCF and adjacent townships were also reported in China (Weng *et al.*, 2007) based on VNTR analysis which led them to postulate that the trade fairs may be a possible means of transmission. In the present study also, sample pairs 12 and 19, 32 and 21 share similar VNTR patterns at 14 of the loci. Similarly, sample pairs 3 and 33, 3 and 19, 4 and 30, 36 and 21, 38 and 19 share similar VNTR patterns at 13 of the loci. However, epidemiological links between these sample pairs remain obscure.

6.2 Conclusion

Globally, studies on molecular epidemiology of leprosy have been based on VNTR polymorphisms detected by methods like DNA fragment analysis and sequencing. Nepal, although being an endemic country for leprosy, lacks accurate data on strain types to compare with global data due to crude methods previously used for such type of study. This study has been successful in establishing the DNA fragment analysis as a method for studying polymorphisms at VNTR loci in Nepal. DNA fragment analysis is a better way of estimating the correct amplicon sizes which is vital in studying polymorphisms at VNTR loci. Similarly, the study also emphasizes multiplex-PCR as a time-efficient method for such types of study.

Review of the published data on *M. leprae* VNTR has demonstrated variability in VNTR loci to be characteristic of different countries and region. Not all VNTR markers are equally variable in all the regions. Markers need to be determined empirically for each population or region of endemicity (Sakamuri *et al.*, 2009b). Although the sample size was insufficient for statistical significance, initial sampling indicates that diversity at *rpoT* may not be considerably high among Nepali population. Combination of less discriminatory loci (23-3, AC8b, 12-5 and 27-5) and moderately discriminatory loci (GGT5, 21-3, ML01 and 6-7) may be employed to characterize *M. leprae* strains from Nepal as a whole. When a region-wise resolution of *M. leprae* is sought, highly discriminatory loci (which are usually microsatellite loci) can be added to the combination of less and moderately discriminatory loci.

The study was carried out with only 40 samples which is quite a small sample to effectively extrapolate the results to whole Nepali population. However, the results obtained from this study are generally comparative to what has been found in other countries like India and China in terms of allele diversity at VNTR loci studied. Within the limited sample size, no association between the strain types and geographical distribution and reactionary outcome were observed.

Chapter 7
Summary and Recommendations

7.1 Summary

Slit skin scrapings from 40 MB leprosy patient were collected for of *M. leprae* DNA extraction. Extracted DNA was then employed for multiplex PCR amplification of 18 VNTR loci. The VNTR loci included 10 microsatellite loci and 8 minisatellite loci. MLVA was performed based on the data obtained from DNA fragment analysis. The *rpoT* locus alone was invariable among the 40 samples from Nepalese population. 23-3 and (AC)8b were similarly less polymorphic. Minisatellite loci were comparably less polymorphic than microsatellite loci. 6-7 was the most polymorphic among minisatellite loci with diversity index 0.58. (TA)18 was the most polymorphic of all with diversity index 0.93. Similar VNTR patterns were observed in the patients of the same family which indicates the utility of MLVA in assessing transmission linkages. In general, the study indicates the prevalence of diverse genotypes of *M. leprae* circulating within Nepalese population based on VNTR profiles studied using DNA fragment analysis. In summary, SSS can be used along with multiplex PCR and FLA in Nepal in order to determine VNTR strain typing according to international starndards.

7.2 Recommendations

This study aimed to establish a standard method to correctly interpret data on VNTR analysis for *M. leprae* strain differentiation in Nepal. During its operation, a few practical limitations were observed which could be addressed in future studies. Recommendations made from this research work are as follows:

1. In general, minisatellite loci were less polymorphic and microsatellite loci were more polymorphic. When greater resolution of *M. leprae* strains within Nepal is sought, both kinds should be incorporated.
2. DNA fragment analysis can and should be performed when conducting MLVA studies in Nepal in order to corroborate with global leprosy reporting networks.
3. The 18-8 locus seemed to have problems with amplification within this group of samples using this procedure. Null alleles could be observed in many of the

samples. Incorporation of a DNA purification protocol should to tried in order to potentially minimize the problem.

4. Stutter phenomena and 3'-end tailing effects are common drawbacks when analyzing microsatellite loci. Such phenomena make interpretation of data difficult. Future studies should consider minimizing such effects.
5. Multiple bands on electropherograms could be observed which may raise questions of infection with multiple strains. A more systematic method of interpretation should be sought to decide whether to include or exclude such possibilities of multiple infection.
6. VNTR-FLA analysis should be performed using Nepalese leprosy isolates in order to maintain an epidemiological database of detected strain types from broader areas across Nepal. This would be useful for connecting cases or demonstrating reinfection or relapse in future.

Chapter 8
References

Abulafia, J., and Vignale, R.A. (1999) Leprosy: pathogenesis updated. *Int J Dermatol* **38**: 321-334.

Achilles, E.G., Hagel, C., Vierbuchen, M., and Dietrich, M. (2004) Leprosy accidentally transmitted from a patient to a surgeon in a nonendemic area. *Ann Intern Med* **141**: W51.

Adams, L.B., Job, C.K., and Krahenbuhl, J.L. (2000) Role of inducible nitric oxide synthase in resistance to Mycobacterium leprae in mice. *Infect Immun* **68**: 5462-5465.

Adhikari, B. (2009) Use of mionisatellite genetic profiling with clinical analysis of leprosy patients in Nepal. A dissertation submitted to Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Allavena, P., Chieppa, M., Monti, P., and Piemonti, L. (2004) From pattern recognition receptor to regulator of homeostasis: the double-faced macrophage mannose receptor. *Crit Rev Immunol* **24**: 179-192.

Anderson, R.P., and Roth, J.R. (1977) Tandem genetic duplications in phage and bacteria. *Annu Rev Microbiol* **31**: 473-505.

Araoz, R., Honore, N., Banu, S., Demangel, C., Cissoko, Y., Arama, C., Uddin, M.K., Hadi, S.K., Monot, M., Cho, S.N., Ji, B., Brennan, P.J., Sow, S., and Cole, S.T. (2006) Towards an immunodiagnostic test for leprosy. *Microbes Infect* **8**: 2270-2276.

Banerjee, S., Ray, D., Bandyopadhyay, D., Gupta, S., Gupta, S., Ghosal, C., Biswas, N., Bhattacharya, S., Dutta, R.N., and Bhattacharya, B. (2008) Development and

application of a new efficient and sensitive multiplex polymerase chain reaction (PCR) in diagnosis of leprosy. *J Indian Med Assoc* **106**: 436-440.

Bang, P.D., Suzuki, K., Phuong le, T., Chu, T.M., Ishii, N., and Khang, T.H. (2009) Evaluation of polymerase chain reaction-based detection of Mycobacterium leprae for the diagnosis of leprosy. *J Dermatol* **36**: 269-276.

Barker, D.J.P., and Hall, A.J. (1991) *Practical epidemiology*. Edinburgh: Churchill Livingstone; Pp : 57

Becx-Bleumink, M., and Berhe, D. (1992) Occurrence of reactions, their diagnosis and management in leprosy patients treated with multidrug therapy; experience in the leprosy control program of the All Africa Leprosy and Rehabilitation Training Center (ALERT) in Ethiopia. *Int J Lepr Other Mycobact Dis* **60**: 173-184.

Berg, I., Neumann, R., Cederberg, H., Rannug, U., and Jeffreys, A.J. (2003) Two modes of germline instability at human minisatellite MS1 (locus D1S7): complex rearrangements and paradoxical hyperdeletion. *Am J Hum Genet* **72**: 1436-1447.

Blake, L.A., West, B.C., Lary, C.H., and Todd, J.R.t. (1987) Environmental nonhuman sources of leprosy. *Rev Infect Dis* **9**: 562-577.

Bochud, P.Y., Hawn, T.R., and Aderem, A. (2003) Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. *J Immunol* **170**: 3451-3454.

Brandsma, J.W., Bizuneh, E., Temam, F., and Naafs, B. (2008) Mycobacterial infections causing cutaneous disease; or how is leprosy transmitted? *Lepr Rev* **79**: 196-198.

Brennan, P.J. (2000) Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. *Lepr Rev* **71 Suppl**: S50-54.

Britten, R.J., and Kohne, D.E. (1968) Repeated sequences in DNA. Hundreds of thousands of copies of DNA sequences have been incorporated into the genomes of higher organisms. *Science* **161**: 529-540.

Britton, W.J., and Lockwood, D.N. (2004) Leprosy. *Lancet* **363**: 1209-1219.

Buhrer-Sekula, S., Smits, H.L., Gussenhoven, G.C., van Leeuwen, J., Amador, S., Fujiwara, T., Klatser, P.R., and Oskam, L. (2003) Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. *J Clin Microbiol* **41**: 1991-1995.

Buhrer, S.S., Smits, H.L., Gussenhoven, G.C., van Ingen, C.W., and Klatser, P.R. (1998) A simple dipstick assay for the detection of antibodies to phenolic glycolipid-I of Mycobacterium leprae. *Am J Trop Med Hyg* **58**: 133-136.

Cardona-Castro, N., Beltran-Alzate, J.C., Romero-Montoya, I.M., Melendez, E., Torres, F., Sakamuri, R.M., Li, W., and Vissa, V. (2009a) Identification and comparison of Mycobacterium leprae genotypes in two geographical regions of Colombia. *Lepr Rev* **80**: 316-321.

Cardona-Castro, N., Beltran-Alzate, J.C., and Romero-Montoya, M. (2009b) Clinical, bacteriological and immunological follow-up of household contacts of leprosy patients from a post-elimination area - Antioquia, Colombia. *Mem Inst Oswaldo Cruz* **104**: 935-936.

Clark-Curtiss, J.E., and Walsh, G.P. (1989) Conservation of genomic sequences among isolates of Mycobacterium leprae. *J Bacteriol* **171**: 4844-4851.

Clark, D.P., and Russell, L.D. (2005) *Molecular biology : made simple and fun.* St. Louis, MO: Cache River Press.

Cole, S.T., Eiglmeier, K., Parkhill, J., James, K.D., Thomson, N.R., Wheeler, P.R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R.M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J.R., and Barrell, B.G. (2001a) Massive gene decay in the leprosy bacillus. *Nature* **409**: 1007-1011.

Cole, S.T., Supply, P., and Honore, N. (2001b) Repetitive sequences in Mycobacterium leprae and their impact on genome plasticity. *Lepr Rev* **72**: 449-461.

Colston, M.J., and Hilson, G.R. (1976) Growth of Mycobacterium leprae and M. marinum in congenitally athymic (nude) mice. *Nature* **262**: 399-401.

Cox, R., and Mirkin, S.M. (1997) Characteristic enrichment of DNA repeats in different genomes. *Proc Natl Acad Sci U S A* **94**: 5237-5242.

Dakin, E.E., and Avise, J.C. (2004) Microsatellite null alleles in parentage analysis. *Heredity* **93**: 504-509.

de Wit, M.Y., Douglas, J.T., McFadden, J., and Klatser, P.R. (1993) Polymerase chain reaction for detection of Mycobacterium leprae in nasal swab specimens. *J Clin Microbiol* **31**: 502-506.

de Wit, M.Y., and Klatser, P.R. (1994) Mycobacterium leprae isolates from different sources have identical sequences of the spacer region between the 16S and 23S ribosomal RNA genes. *Microbiology* **140 (Pt 8)**: 1983-1987.

Demangel, C., and Britton, W.J. (2000) Interaction of dendritic cells with mycobacteria: where the action starts. *Immunol Cell Biol* **78**: 318-324.

Deps, P.D., Alves, B.L., Gripp, C.G., Aragao, R.L., Guedes, B., Filho, J.B., Andreatta, M.K., Marcari, R.S., Prates, I., and Rodrigues, L.C. (2008) Contact with armadillos increases the risk of leprosy in Brazil: a case control study. *Indian J Dermatol Venereol Leprol* **74**: 338-342.

Desikan, K.V., and Sreevatsa (1995) Extended studies on the viability of Mycobacterium leprae outside the human body. *Lepr Rev* **66**: 287-295.

Douglas, J.T., and Worth, R.M. (1984) Field evaluation of an ELISA to detect antibody in leprosy patients and their contacts. *Int J Lepr Other Mycobact Dis* **52**: 26-33.

Duraes, S.M., Guedes, L.S., Cunha, M.D., Magnanini, M.M., and Oliveira, M.L. (2010) Epidemiologic study of 107 cases of families with leprosy in Duque de Caxias, Rio de Janeiro, Brazil. *An Bras Dermatol* **85**: 339-345.

Edwards, A., Civitello, A., Hammond, H.A., and Caskey, C.T. (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet* **49**: 746-756.

Eiglmeier, K., Parkhill, J., Honore, N., Garnier, T., Tekaia, F., Telenti, A., Klatser, P., James, K.D., Thomson, N.R., Wheeler, P.R., Churcher, C., Harris, D., Mungall, K., Barrell, B.G., and Cole, S.T. (2001) The decaying genome of Mycobacterium leprae. *Lepr Rev* **72**: 387-398.

Ellegren, H. (2004) Microsatellites: simple sequences with complex evolution. *Nat Rev Genet* **5**: 435-445.

Feuth, M., Brandsma, J.W., Faber, W.R., Bhattarai, B., Feuth, T., and Anderson, A.M. (2008) Erythema nodosum leprosum in Nepal: a retrospective study of clinical

features and response to treatment with prednisolone or thalidomide. *Lepr Rev* **79**: 254-269.

Fine, P.E., Sterne, J.A., Ponnighaus, J.M., Bliss, L., Saui, J., Chihana, A., Munthali, M., and Warndorff, D.K. (1997) Household and dwelling contact as risk factors for leprosy in northern Malawi. *Am J Epidemiol* **146**: 91-102.

Fontes, A.N., Sakamuri, R.M., Baptista, I.M., Ura, S., Moraes, M.O., Martinez, A.N., Sarno, E.N., Brennan, P.J., Vissa, V.D., and Suffys, P.N. (2009) Genetic diversity of mycobacterium leprae isolates from Brazilian leprosy patients. *Lepr Rev* **80**: 302-315.

Fowler, J.C., Burgoyne, L.A., Scott, A.C., and Harding, H.W. (1988) Repetitive deoxyribonucleic acid (DNA) and human genome variation--a concise review relevant to forensic biology. *J Forensic Sci* **33**: 1111-1126.

Frothingham, R., and Meeker-O'Connell, W.A. (1998) Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. *Microbiology* **144 (Pt 5)**: 1189-1196.

Fsihi, H., and Cole, S.T. (1995) The Mycobacterium leprae genome: systematic sequence analysis identifies key catabolic enzymes, ATP-dependent transport systems and a novel polA locus associated with genomic variability. *Mol Microbiol* **16**: 909-919.

Geluk, A., van der Ploeg, J., Teles, R.O., Franken, K.L., Prins, C., Drijfhout, J.W., Sarno, E.N., Sampaio, E.P., and Ottenhoff, T.H. (2008) Rational combination of peptides derived from different Mycobacterium leprae proteins improves sensitivity for immunodiagnosis of M. leprae infection. *Clin Vaccine Immunol* **15**: 522-533.

Ghorpade, A. (2002) Inoculation (tattoo) leprosy: a report of 31 cases. *J Eur Acad Dermatol Venereol* **16**: 494-499.

Ghorpade, A. (2009a) Ornamental tattoos and skin lesions. Tattoo inoculation borderline tuberculoid leprosy. *Int J Dermatol* **48**: 11-13.

Ghorpade, A. (2009b) Post-traumatic inoculation tuberculoid leprosy after injury with a glass bangle. *Lepr Rev* **80**: 215-218.

Gillis, T., Vissa, V., Matsuoka, M., Young, S., Richardus, J.H., Truman, R., Hall, B., and Brennan, P. (2009) Characterisation of short tandem repeats for genotyping Mycobacterium leprae. *Lepr Rev* **80**: 250-260.

Gilson, E., Perrin, D., Saurin, W., and Hofnung, M. (1987) Species specificity of bacterial palindromic units. *J Mol Evol* **25**: 371-373.

Gordon, S. (2004) Pathogen recognition or homeostasis? APC receptor functions in innate immunity. *C R Biol* **327**: 603-607.

Grange, J.M., Dewar, C.A., and Rowbotham, T.J. (1987) Microbe dependence of Mycobacterium leprae: a possible intracellular relationship with protozoa. *Int J Lepr Other Mycobact Dis* **55**: 565-566.

Groathouse, N.A., Rivoire, B., Kim, H., Lee, H., Cho, S.N., Brennan, P.J., and Vissa, V.D. (2004) Multiple polymorphic loci for molecular typing of strains of Mycobacterium leprae. *J Clin Microbiol* **42**: 1666-1672.

Hagge, D.A., Ray, N.A., Krahenbuhl, J.L., and Adams, L.B. (2004) An in vitro model for the lepromatous leprosy granuloma: fate of Mycobacterium leprae from target macrophages after interaction with normal and activated effector macrophages. *J Immunol* **172**: 7771-7779.

Hagge, D.A., Marks, V.T., Ray, N.A., Dietrich, M.A., Kearney, M.T., Scollard, D.M., Krahenbuhl, J.L., and Adams, L.B. (2007) Emergence of an effective adaptive cell mediated immune response to Mycobacterium leprae is not impaired in

reactive oxygen intermediate-deficient mice. *FEMS Immunol Med Microbiol* **51**: 92-101.

Hartl, D.L., and Jones, E.W. (2009) *Genetics : analysis of genes and genomes.* Sudbury, Mass.: Jones and Bartlett Publishers.; Pp : 67

Hartskeerl, R.A., de Wit, M.Y., and Klatser, P.R. (1989) Polymerase chain reaction for the detection of Mycobacterium leprae. *J Gen Microbiol* **135**: 2357-2364.

Hashimoto, K., Maeda, Y., Kimura, H., Suzuki, K., Masuda, A., Matsuoka, M., and Makino, M. (2002) Mycobacterium leprae infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. *Infect Immun* **70**: 5167-5176.

Hastings, R.C. (1985) *Leprosy*: Churchill Livingstone; Pp : 31, 33, 53-63

Hastings, R.C., Gillis, T.P., Krahenbuhl, J.L., and Franzblau, S.G. (1988) Leprosy. *Clin Microbiol Rev* **1**: 330-348.

Hatfull, G.F., and Jacobs, W.R. (2000) *Molecular genetics of mycobacteria.* Washington, D.C.: ASM Press.; Pp : 23

Jacob, H.J., Lindpaintner, K., Lincoln, S.E., Kusumi, K., Bunker, R.K., Mao, Y.P., Ganten, D., Dzau, V.J., and Lander, E.S. (1991) Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. *Cell* **67**: 213-224.

Jacobson, R.R., and Krahenbuhl, J.L. (1999) Leprosy. *Lancet* **353**: 655-660.

Jeffreys, A.J., Wilson, V., and Thein, S.L. (1985) Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**: 67-73.

Job, C.K., Jayakumar, J., Kearney, M., and Gillis, T.P. (2008) Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. *Am J Trop Med Hyg* **78**: 518-521.

Kahawita, I.P., and Lockwood, D.N. (2008) Towards understanding the pathology of erythema nodosum leprosum. *Trans R Soc Trop Med Hyg* **102**: 329-337.

Kang, T.J., and Chae, G.T. (2001) Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. *FEMS Immunol Med Microbiol* **31**: 53-58.

Keim, P., Price, L.B., Klevytska, A.M., Smith, K.L., Schupp, J.M., Okinaka, R., Jackson, P.J., and Hugh-Jones, M.E. (2000) Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within Bacillus anthracis. *J Bacteriol* **182**: 2928-2936.

Kimura, M., Sakamuri, R.M., Groathouse, N.A., Rivoire, B.L., Gingrich, D., Krueger-Koplin, S., Cho, S.N., Brennan, P.J., and Vissa, V. (2009) Rapid variable-number tandem-repeat genotyping for Mycobacterium leprae clinical specimens. *J Clin Microbiol* **47**: 1757-1766.

Klatser, P.R., van Beers, S., Madjid, B., Day, R., and de Wit, M.Y. (1993) Detection of Mycobacterium leprae nasal carriers in populations for which leprosy is endemic. *J Clin Microbiol* **31**: 2947-2951.

Klevytska, A.M., Price, L.B., Schupp, J.M., Worsham, P.L., Wong, J., and Keim, P. (2001) Identification and characterization of variable-number tandem repeats in the Yersinia pestis genome. *J Clin Microbiol* **39**: 3179-3185.

Koppel, E.A., Ludwig, I.S., Hernandez, M.S., Lowary, T.L., Gadikota, R.R., Tuzikov, A.B., Vandenbroucke-Grauls, C.M., van Kooyk, Y., Appelmelk, B.J., and Geijtenbeek, T.B. (2004) Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. *Immunobiology* **209**: 117-127.

Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J.J. (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res* **18**: 999-1005.

Lahiri, R., and Krahenbuhl, J.L. (2008) The role of free-living pathogenic amoeba in the transmission of leprosy: a proof of principle. *Lepr Rev* **79**: 401-409.

Lavania, M., Katoch, K., Katoch, V.M., Gupta, A.K., Chauhan, D.S., Sharma, R., Gandhi, R., Chauhan, V., Bansal, G., Sachan, P., Sachan, S., Yadav, V.S., and Jadhav, R. (2008) Detection of viable Mycobacterium leprae in soil samples: insights into possible sources of transmission of leprosy. *Infect Genet Evol* **8**: 627-631.

Le Fleche, P., Hauck, Y., Onteniente, L., Prieur, A., Denoeud, F., Ramisse, V., Sylvestre, P., Benson, G., Ramisse, F., and Vergnaud, G. (2001) A tandem repeats database for bacterial genomes: application to the genotyping of Yersinia pestis and Bacillus anthracis. *BMC Microbiol* **1**: 2.

Lini, N., Shankernarayan, N.P., and Dharmalingam, K. (2009) Quantitative real-time PCR analysis of Mycobacterium leprae DNA and mRNA in human biopsy material from leprosy and reactional cases. *J Med Microbiol* **58**: 753-759.

Litt, M., and Luty, J.A. (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet* **44**: 397-401.

Lockwood, D.N., and Sinha, H.H. (1999) Pregnancy and leprosy: a comprehensive literature review. *Int J Lepr Other Mycobact Dis* **67**: 6-12.

Maeda, Y., Gidoh, M., Ishii, N., Mukai, C., and Makino, M. (2003) Assessment of cell mediated immunogenicity of Mycobacterium leprae-derived antigens. *Cell Immunol* **222**: 69-77.

Maiden, M.C. (2009) Putting leprosy on the map. *Nat Genet* **41**: 1264-1266.

Malorny, B., Junker, E., and Helmuth, R. (2008) Multi-locus variable-number tandem repeat analysis for outbreak studies of Salmonella enterica serotype Enteritidis. *BMC Microbiol* **8**: 84.

Manandhar, R., LeMaster, J.W., and Roche, P.W. (1999) Risk factors for erythema nodosum leprosum. *Int J Lepr Other Mycobact Dis* **67**: 270-278.

Marshall, D.G., Coleman, D.C., Sullivan, D.J., Xia, H., O'Morain, C.A., and Smyth, C.J. (1996) Genomic DNA fingerprinting of clinical isolates of Helicobacter pylori using short oligonucleotide probes containing repetitive sequences. *J Appl Bacteriol* **81**: 509-517.

Martinez, A.N., Britto, C.F., Nery, J.A., Sampaio, E.P., Jardim, M.R., Sarno, E.N., and Moraes, M.O. (2006) Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of Mycobacterium leprae DNA in skin biopsy samples from patients diagnosed with leprosy. *J Clin Microbiol* **44**: 3154-3159.

Matsuoka, M., Izumi, S., Budiawan, T., Nakata, N., and Saeki, K. (1999) Mycobacterium leprae DNA in daily using water as a possible source of leprosy infection. *Indian J Lepr* **71**: 61-67.

Matsuoka, M., Maeda, S., Kai, M., Nakata, N., Chae, G.T., Gillis, T.P., Kobayashi, K., Izumi, S., and Kashiwabara, Y. (2000) Mycobacterium leprae typing by genomic diversity and global distribution of genotypes. *Int J Lepr Other Mycobact Dis* **68**: 121-128.

Matsuoka, M., Zhang, L., Budiawan, T., Saeki, K., and Izumi, S. (2004) Genotyping of Mycobacterium leprae on the basis of the polymorphism of TTC repeats for analysis of leprosy transmission. *J Clin Microbiol* **42**: 741-745.

Matsuoka, M. (2009) Recent advances in the molecular epidemiology of leprosy. *Nihon Hansenbyo Gakkai Zasshi* **78**: 67-73.

Meyers, W.M., Gormus, B.J., and Walsh, G.P. (1992) Nonhuman sources of leprosy. *Int J Lepr Other Mycobact Dis* **60**: 477-480.

Misra, N., Ramesh, V., Misra, R.S., Narayan, N.P., Colston, M.J., and Nath, I. (1995) Clinical utility of LSR/A15 gene for Mycobacterium leprae detection in leprosy tissues using the polymerase chain reaction. *Int J Lepr Other Mycobact Dis* **63**: 35-41.

Mittal, A., Mishra, R.S., and Nath, I. (1989) Accessory cell heterogeneity in lepromatous leprosy; dendritic cells and not monocytes support T cell responses. *Clin Exp Immunol* **76**: 233-239.

Modlin, R.L., Melancon-Kaplan, J., Young, S.M., Pirmez, C., Kino, H., Convit, J., Rea, T.H., and Bloom, B.R. (1988) Learning from lesions: patterns of tissue inflammation in leprosy. *Proc Natl Acad Sci U S A* **85**: 1213-1217.

Modlin, R.L. (1994) Th1-Th2 paradigm: insights from leprosy. *J Invest Dermatol* **102**: 828-832.

MoHP (2007/2008) Annual Report 2007/08. Ministry of Health and Population, Department of Health Services, Leprosy Control Program, Kathmandu, Nepal.

MoHP (2009) Report on Achievement of Elimination of Leproy as a Public Health Problem in Nepal.: Ministry of Health and Population, Department of Health Services, Leprosy Control Division, Teku, Kathmandu.

MoHP (2009/2010) Annual Report 2009/2010. Ministry of Health and Population, Department of Health Services, Leprosy Control Program, Kathmandu, Nepal.

Monot, M., Honore, N., Garnier, T., Araoz, R., Coppee, J.Y., Lacroix, C., Sow, S., Spencer, J.S., Truman, R.W., Williams, D.L., Gelber, R., Virmond, M., Flageul, B., Cho, S.N., Ji, B., Paniz-Mondolfi, A., Convit, J., Young, S., Fine, P.E., Rasolofo, V., Brennan, P.J., and Cole, S.T. (2005) On the origin of leprosy. *Science* **308**: 1040-1042.

Monot, M., Honore, N., Garnier, T., Zidane, N., Sherafi, D., Paniz-Mondolfi, A., Matsuoka, M., Taylor, G.M., Donoghue, H.D., Bouwman, A., Mays, S., Watson, C., Lockwood, D., Khamesipour, A., Dowlati, Y., Jianping, S., Rea, T.H., Vera-Cabrera, L., Stefani, M.M., Banu, S., Macdonald, M., Sapkota, B.R., Spencer, J.S., Thomas, J., Harshman, K., Singh, P., Busso, P., Gattiker, A., Rougemont, J., Brennan, P.J., and Cole, S.T. (2009) Comparative genomic and phylogeographic analysis of Mycobacterium leprae. *Nat Genet* **41**: 1282-1289.

Moura, R.S., Calado, K.L., Oliveira, M.L., and Buhrer-Sekula, S. (2008) Leprosy serology using PGL-I: a systematic review. *Rev Soc Bras Med Trop* **41 Suppl 2**: 11-18.

Nederbragt, A.J., Balasingham, A., Sirevag, R., Utkilen, H., Jakobsen, K.S., and Anderson-Glenna, M.J. (2008) Multiple-locus variable-number tandem repeat analysis of Legionella pneumophila using multi-colored capillary electrophoresis. *J Microbiol Methods* **73**: 111-117.

Noordeen, S.K. (1994) Elimination of leprosy as a public health problem. *Int J Lepr Other Mycobact Dis* **62**: 278-283.

Pandey, S. (2005) Genetic variability of Mycobacterium leprae in Nepal. A dissertationsubmitted to the Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Plikaytis, B.B., Gelber, R.H., and Shinnick, T.M. (1990) Rapid and sensitive detection of Mycobacterium leprae using a nested-primer gene amplification assay. *J Clin Microbiol* **28**: 1913-1917.

Pocaterra, L., Jain, S., Reddy, R., Muzaffarullah, S., Torres, O., Suneetha, S., and Lockwood, D.N. (2006) Clinical course of erythema nodosum leprosum: an 11-year cohort study in Hyderabad, India. *Am J Trop Med Hyg* **74**: 868-879.

Prodinger, W.M., Brandstatter, A., Naumann, L., Pacciarini, M., Kubica, T., Boschiroli, M.L., Aranaz, A., Nagy, G., Cvetnic, Z., Ocepek, M., Skrypnyk, A., Erler, W., Niemann, S., Pavlik, I., and Moser, I. (2005) Characterization of Mycobacterium caprae isolates from Europe by mycobacterial interspersed repetitive unit genotyping. *J Clin Microbiol* **43**: 4984-4992.

Ranque, B., Nguyen, V.T., Vu, H.T., Nguyen, T.H., Nguyen, N.B., Pham, X.K., Schurr, E., Abel, L., and Alcais, A. (2007) Age is an important risk factor for onset and sequelae of reversal reactions in Vietnamese patients with leprosy. *Clin Infect Dis* **44**: 33-40.

Rees, R.J., Waters, M.F., Weddell, A.G., and Palmer, E. (1967) Experimental lepromatous leprosy. *Nature* **215**: 599-602.

Rees, R.J., and McDougall, A.C. (1977) Airborne infection with Mycobacterium leprae in mice. *J Med Microbiol* **10**: 63-68.

Ridley, D.S., and Jopling, W.H. (1966) Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* **34**: 255-273.

Riley, L.W. (2004) *Molecular epidemiology of infectious diseases : principles and practices.* Washington, D.C.: ASM Press.

Robertson, B.D., and Meyer, T.F. (1992) Genetic variation in pathogenic bacteria. *Trends Genet* **8**: 422-427.

Roche, P.W., Theuvenet, W.J., and Britton, W.J. (1991) Risk factors for type-1 reactions in borderline leprosy patients. *Lancet* **338**: 654-657.

Rudeeaneksin, J., Srisungngam, S., Sawanpanyalert, P., Sittiwakin, T., Likanonsakul, S., Pasadorn, S., Palittapongarnpim, P., Brennan, P.J., and Phetsuksiri, B. (2008) LightCycler real-time PCR for rapid detection and quantitation of Mycobacterium leprae in skin specimens. *FEMS Immunol Med Microbiol* **54**: 263-270.

Sakamuri, R.M., Harrison, J., Gelber, R., Saunderson, P., Brennan, P.J., Balagon, M., and Vissa, V. (2009a) A continuation: study and characterisation of Mycobacterium leprae short tandem repeat genotypes and transmission of leprosy in Cebu, Philippines. *Lepr Rev* **80**: 272-279.

Sakamuri, R.M., Kimura, M., Li, W., Kim, H.C., Lee, H., Kiran, M.D., Black, W.C.t., Balagon, M., Gelber, R., Cho, S.N., Brennan, P.J., and Vissa, V. (2009b) Population-based molecular epidemiology of leprosy in Cebu, Philippines. *J Clin Microbiol* **47**: 2844-2854.

Sambrook, J., Russell, D.W., and Cold Spring Harbor Laboratory. (2001) *Molecular cloning : a laboratory manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Santos, D.O., Santos, S.L., Esquenazi, D., Nery, J.A., Defruyt, M., Lorre, K., and Van Heuverswyn, H. (2001) Evaluation of B7-1 (CD80) and B7-2 (CD86) costimulatory molecules and dendritic cells on the immune response in leprosy. *Nihon Hansenbyo Gakkai Zasshi* **70**: 15-24.

Schlesinger, L.S., and Horwitz, M.A. (1991) Phagocytosis of Mycobacterium leprae by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacterium. *J Immunol* **147**: 1983-1994.

Scollard, D.M., Adams, L.B., Gillis, T.P., Krahenbuhl, J.L., Truman, R.W., and Williams, D.L. (2006) The continuing challenges of leprosy. *Clin Microbiol Rev* **19**: 338-381.

Sekar, B. (2007) Recent advances in immunodiagnosis of leprosy. *Indian J Lepr* **79**: 85-106.

Shin, Y.C., Lee, H., Lee, H., Walsh, G.P., Kim, J.D., and Cho, S.N. (2000) Variable numbers of TTC repeats in Mycobacterium leprae DNA from leprosy patients and use in strain differentiation. *J Clin Microbiol* **38**: 4535-4538.

Shinde, V., Newton, H., Sakamuri, R.M., Reddy, V., Jain, S., Joseph, A., Gillis, T., Nath, I., Norman, G., and Vissa, V. (2009) VNTR typing of Mycobacterium leprae in South Indian leprosy patients. *Lepr Rev* **80**: 290-301.

Sieling, P.A., Jullien, D., Dahlem, M., Tedder, T.F., Rea, T.H., Modlin, R.L., and Porcelli, S.A. (1999) CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. *J Immunol* **162**: 1851-1858.

Skuce, R.A., McCorry, T.P., McCarroll, J.F., Roring, S.M., Scott, A.N., Brittain, D., Hughes, S.L., Hewinson, R.G., and Neill, S.D. (2002) Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. *Microbiology* **148**: 519-528.

Smith, C., and Richardus, J.H. (2008) Leprosy strategy is about control, not eradication. *Lancet* **371**: 969-970.

Smith, W.C., Smith, C.M., Cree, I.A., Jadhav, R.S., Macdonald, M., Edward, V.K., Oskam, L., van Beers, S., and Klatser, P. (2004) An approach to understanding the transmission of Mycobacterium leprae using molecular and immunological methods: results from the MILEP2 study. *Int J Lepr Other Mycobact Dis* **72**: 269-277.

Sola, C., Filliol, I., Legrand, E., Lesjean, S., Locht, C., Supply, P., and Rastogi, N. (2003) Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infect Genet Evol* **3**: 125-133.

Spurgiesz, R.S., Quitugua, T.N., Smith, K.L., Schupp, J., Palmer, E.G., Cox, R.A., and Keim, P. (2003) Molecular typing of Mycobacterium tuberculosis by using nine novel variable-number tandem repeats across the Beijing family and low-copy-number IS6110 isolates. *J Clin Microbiol* **41**: 4224-4230.

Srisungnam, S., Rudeeaneksin, J., Lukebua, A., Wattanapokayakit, S., Pasadorn, S., Mahotarn, K., Ajincholapan, Sakamuri, R.M., Kimura, M., Brennan, P.J., Phetsuksiri, B., and Vissa, V. (2009) Molecular epidemiology of leprosy based on VNTR typing in Thailand. *Lepr Rev* **80**: 280-289.

Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B., and Locht, C. (2000) Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. *Mol Microbiol* **36**: 762-771.

Takade, A., Umeda, A., Matsuoka, M., Yoshida, S., Nakamura, M., and Amako, K. (2003) Comparative studies of the cell structures of Mycobacterium leprae and M. tuberculosis using the electron microscopy freeze-substitution technique. *Microbiol Immunol* **47**: 265-270.

Truman, R., Fontes, A.B., De Miranda, A.B., Suffys, P., and Gillis, T. (2004) Genotypic variation and stability of four variable-number tandem repeats and their suitability for discriminating strains of Mycobacterium leprae. *J Clin Microbiol* **42**: 2558-2565.

Truman, R., and Fine, P.E. (2010) 'Environmental' sources of Mycobacterium leprae: issues and evidence. *Lepr Rev* **81**: 89-95.

Truman, R.W., and Krahenbuhl, J.L. (2001) Viable M. leprae as a research reagent. *Int J Lepr Other Mycobact Dis* **69**: 1-12.

Truman, R.W., Andrews, P.K., Robbins, N.Y., Adams, L.B., Krahenbuhl, J.L., and Gillis, T.P. (2008) Enumeration of Mycobacterium leprae using real-time PCR. *PLoS Negl Trop Dis* **2**: e328.

van Belkum, A., Scherer, S., van Leeuwen, W., Willemse, D., van Alphen, L., and Verbrugh, H. (1997) Variable number of tandem repeats in clinical strains of Haemophilus influenzae. *Infect Immun* **65**: 5017-5027.

van Belkum, A., Scherer, S., van Alphen, L., and Verbrugh, H. (1998) Short-sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev* **62**: 275-293.

van Belkum, A. (1999) Short sequence repeats in microbial pathogenesis and evolution. *Cell Mol Life Sci* **56**: 729-734.

Van Brakel, W.H., Khawas, I.B., and Lucas, S.B. (1994) Reactions in leprosy: an epidemiological study of 386 patients in west Nepal. *Lepr Rev* **65**: 190-203.

van Kooyk, Y., and Geijtenbeek, T.B. (2003) DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol* **3**: 697-709.

Varkevisser, C.M., Lever, P., Alubo, O., Burathoki, K., Idawani, C., Moreira, T.M., Patrobas, P., and Yulizar, M. (2009) Gender and leprosy: case studies in Indonesia, Nigeria, Nepal and Brazil. *Lepr Rev* **80**: 65-76.

Vissa, V.D., and Brennan, P.J. (2001) The genome of Mycobacterium leprae: a minimal mycobacterial gene set. *Genome Biol* **2**: REVIEWS1023.

Walker, S.L., and Lockwood, D.N. (2006) The clinical and immunological features of leprosy. *Br Med Bull* **77-78**: 103-121.

Walker, S.L., and Lockwood, D.N. (2007) Leprosy. *Clin Dermatol* **25**: 165-172.

Walsh, G.P., Meyers, W.M., Binford, C.H., Gormus, B.J., Baskin, G.B., Wolf, R.H., and Gerone, P.J. (1988) Leprosy as a zoonosis: an update. *Acta Leprol* **6**: 51-60.

Wang, Y.W., Watanabe, H., Phung, D.C., Tung, S.K., Lee, Y.S., Terajima, J., Liang, S.Y., and Chiou, C.S. (2009) Multilocus variable-number tandem repeat analysis for molecular typing and phylogenetic analysis of Shigella flexneri. *BMC Microbiol* **9**: 278.

Weir, R.E., Brennan, P.J., Butlin, C.R., and Dockrell, H.M. (1999) Use of a whole blood assay to evaluate in vitro T cell responses to new leprosy skin test antigens in leprosy patients and healthy subjects. *Clin Exp Immunol* **116**: 263-269.

Weng, X., Wang, Z., Liu, J., Kimura, M., Black, W.C.t., Brennan, P.J., Li, H., and Vissa, V.D. (2007) Identification and distribution of Mycobacterium leprae genotypes in a region of high leprosy prevalence in China: a 3-year molecular epidemiological study. *J Clin Microbiol* **45**: 1728-1734.

Weng, X.M., Wen, Y., Tian, X.J., Wang, H.B., Tan, X.J., and Li, H.Y. (2006) [Preliminary study on the genotyping of Mycobacterium leprae on 50 isolates from China]. *Zhonghua Liu Xing Bing Xue Za Zhi* **27**: 402-405.

Wheeler, P.R. (2001) The microbial physiologist's guide to the leprosy genome. *Lepr Rev* **72**: 399-407.

WHO (1994) Chemotherapy of leprosy. Technical Report Series 847. World Health Organization.

WHO (2003) *Manual of Basic Techniques for Health Laboratory Second Edition*: World Health Organization.

WHO (2009a) Report on the workshop on Sentinel surveillance for Drug Resistance in Leprosy.

WHO (2009b) Weekly Epedimiological Record No. 33 **84**: 333-340.

WHO (2010a) Weekly Epidemilogical Record No. 6. Vol. 85, pp. 37-48.

WHO (2010b) Weekly Epidemilogical Record No. 35 **85**: 337-348.

Williams, D.L., Gillis, T.P., Booth, R.J., Looker, D., and Watson, J.D. (1990a) The use of a specific DNA probe and polymerase chain reaction for the detection of Mycobacterium leprae. *J Infect Dis* **162**: 193-200.

Williams, D.L., Gillis, T.P., and Portaels, F. (1990b) Geographically distinct isolates of Mycobacterium leprae exhibit no genotypic diversity by restriction fragment-length polymorphism analysis. *Mol Microbiol* **4**: 1653-1659.

Woods, S.A., and Cole, S.T. (1989) A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR. *FEMS Microbiol Lett* **53**: 305-309.

Woods, S.A., and Cole, S.T. (1990) A family of dispersed repeats in Mycobacterium leprae. *Mol Microbiol* **4**: 1745-1751.

Xing, Y., Liu, J., Sakamuri, R.M., Wang, Z., Wen, Y., Vissa, V., and Weng, X. (2009) VNTR typing studies of Mycobacterium leprae in China: assessment of methods and stability of markers during treatment. *Lepr Rev* **80**: 261-271.

Yamamura, M., Uyemura, K., Deans, R.J., Weinberg, K., Rea, T.H., Bloom, B.R., and Modlin, R.L. (1991) Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**: 277-279.

Yeramian, E., and Buc, H. (1999) Tandem repeats in complete bacterial genome sequences: sequence and structural analyses for comparative studies. *Res Microbiol* **150**: 745-754.

Young, S.K., Taylor, G.M., Jain, S., Suneetha, L.M., Suneetha, S., Lockwood, D.N., and Young, D.B. (2004) Microsatellite mapping of Mycobacterium leprae populations in infected humans. *J Clin Microbiol* **42**: 4931-4936.

Young, S.K., Ponnighaus, J.M., Jain, S., Lucas, S., Suneetha, S., Lockwood, D.N., Young, D.B., and Fine, P.E. (2008) Use of short tandem repeat sequences to study Mycobacterium leprae in leprosy patients in Malawi and India. *PLoS Negl Trop Dis* **2**: e214.

Zhang, L., Budiawan, T., and Matsuoka, M. (2005) Diversity of potential short tandem repeats in Mycobacterium leprae and application for molecular typing. *J Clin Microbiol* **43**: 5221-5229.

Appendix I

Materials

A. Reagents/Chemicals

Reagents/Chemicals	**Company**
Acetone	Sigma, USA
Acrylamide/bis-acrylamide solution	Sigma, Germany
Ammonium persulphate	Qualigens, India
Boric Acid	Sigma, USA
Bromophenol blue (BPB)	Sigma, USA
Distilled Water	Qualigens, India Sigma, USA
DNA molecular weight marker (100bp)	Banglore Genei, India
EDTA	Sigma, USA
Ethanol	Bengal Chemicals and Pharmaceuticals, India
Ethidium bromide	Sigma, USA
Sodium hypochlorite	Sigma, USA
Sucrose	Sigma, USA
Tetraethyl methylene diamine (TEMED)	Sigma, USA
Multiplex PCR Master Mix	Qiagen, Germany
Q-solution	Qiagen, Germany
Formamide	Applied Biosystems, USA
LIZ-500	Applied Biosystems, USA
Proteinase K	Sigma, USA
Trizma	Sigma, USA
NaCl	Qualigens, India
Na_2HPO_4	Qualigens, India
KH_2PO_4	Qualigens, India
KCl	Qualigens, India
Tween-20	Sigma, USA

B. Glasswares

Beakers (Borosil)
Capillary Cuvette
Conical Flask
Glass rod
Notched glass plate with straight edge
Glass Plates with fixed 1mm spacer
Measuring Cylinder
Glass plate
Pipettes
Test tubes
Volumetric flask (Borosil)
Microcentrifuge tubes

C. Equipment and Apparatus

Equipment	**Company**
Balance	Explorer® Pro, Ohaus, Switzerland
Biological Safety Cabinet Class II	BH12 Labaire System Ltd., UK
Centrifuge	Centrifuge 5415C, Eppendorf, Germany HF120 capsule, Tomy Seiko Ltd., Japan
Dry Heat Block	DB2A , Techne, UK
Electophoresis Unit	PS250 Hybaid, UK
Magnetic Stirrer	Spinstir, Jencons Scientific Ltd., UK
pH meter	pH90 WTW
Polaroid Camera	Polaroid Gelcam, UK
Refrigerator	Express Cool, LG Haier
Spectrophotometer	Biophotometer, Eppendorf, Germany
T3 Thermocycler	Biometra, Germany
UV Trans-illuminator	UVP-Upland, USA
Vertical Gel Electrophoresis Unit	Whatman, Biometra, Germany
Video graphic printer	Sony corporation, Japan
Vortex Shaker	Remi Equipments, India
Genetic Analyzer	ABI310 Genetic Analyzer, Applied Biosystems, USA

D. Pipettes and tubes

Eppendorf tubes — Eppendorf, Germany
Falcon Tubes
Micropipette
(10µl, 20µl, 200µl, 1000µl) — Gilson, France
Micropipette tips — Molecular Bio-product, USA
PCR tubes (0.2 ml) — Molecular Bio-product, USA

E. Miscellaneous

Gloves
Liquid nitrogen
Tissue Paper
Parafilm

Appendix II

Buffers and Reagents Preparation

1. 10X TE buffer (pH 8.0)

100mM Tris-HCl (pH 8.0)
10mM EDTA (pH 8.0)

Sterilize solution by autoclaving at 15psi for 20 mins on liquid cycle. Store at room temperature.

Working Stock, 1X TE (pH 8.0) is prepared by diluting 10 times the original 10X TE (pH 8.0) buffer.

2. 1X PBS buffer (pH 7.4)

137mM	NaCl
10mM	Na_2HPO_4
2mM	KH_2PO_4
2.7mM	KCl

Dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4 in 800 ml of distilled H_2O to make 10X concentration. Adjust the pH to 7.4 with HCl. Add H_2O to 1 litre.

Working Stock, 1X PBS (pH 7.4) is prepared by diluting ten times the 10X PBS (pH 7.4) stock buffer. Working 1X PBS is sterilized by autoclaving for 20 minutes at 15psi on liquid cycle. The buffer is stored at room temperature.

3. Lysis Buffer (pH 8.0)

1mg/ml	Proteinase K
0.1M	Tris-HCl (pH 8.0)
0.05%	Tween-20

To 20ml 0.1M Tris-HCl (pH 8.0), 10µl of Tween-20 is added. 45µl of this buffer solution is aliquoted to separate tubes. To these tubes 5µl Proteinase K (10mg/ml) is added.

4. Tris-Borate EDTA (TBE) buffer (5X) (pH 8.0)

To 500ml Distilled water add

27.25g	Trizma
13.9g	Boric Acid
1.85g	EDTA

TBE is made and stored as a 5X stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer to 1X just before use and make the gel solution and the electrophoresis buffer from the same batch of concentrated stock solution.

Working stock, 1X TBE (pH 8.0) is prepared by diluting five times the original 5X TBE buffer.

5. Bromophenol Blue (BPB) sample buffer

60% w/v Sucrose, 0.25% w/v BPB in TE

Add 30g sucrose to 50ml TE (pH 8.0). Then add 0.025g BPB to 10ml sucrose solution.

6. Ethidium bromide solution

Stock Solution : 10mg/ml of ethidium bromide solution.

Working Solution : To 4720 µl of distilled water, add 280µl of 10mg/ml ethidium bromide solution so that the final concentration is at 500µg/ml.

7. Polyacrylamide Gel (8%) preparation

Distilled Water = 9.486 ml

5X TBE (pH 8.0) = 3.6 ml

30% Acrylamide/bis-acrylamide = 4.788 ml

10% Ammonium persulphate = 126 µl

TEMED = 6.3 µl

Ammonium persulphate is prepared fresh each time.