CHAPTER-I

1. INTRODUCTION

Mycobacterium leprae is an obligate intracellular parasite and the causative agent of leprosy, an infectious disease affecting mainly the skin by invasion of histiocytes and the nerves by invasion of Schwann cells (SCs) (Feuth, *et al.*, 2008). Even though leprosy is one of the oldest recorded disease, the expected elimination of the disease in modern times has been hampered as the underlying mechanisms of transmission of *M. leprae* with respect to the source, reservoir, the portal of its exit/entry and interaction with the host are still unclear (Zhang, *et al.*, 2005; Feuth, *et al.*, 2008). Current theories propose that the nasal mucosa as the potential exit/entry pathway of *M leprae* (Pedley and Geater, 1976; Klatser, *et al.*, 1993; Ramaprasad, *et al.*, 1997); however, field evidence suggests that view as incomplete (Kampirapap, 2008). Multiple transmission modes have been proposed including: human-to-human direct contact (Noordeen, 1994; Fine, *et al.*, 1997), airborne (Smith, *et al.*, 2004), vector-borne (Walsh, *et al.*, 198; Meyers, *et al.*, 1992) and vehicle-borne (Matsuoka, *et al.*, 1999). As untreated leprosy patients represent reservoirs of infection, multidrug therapy (MDT) as designed by the World Health Organization (WHO) was proposed and initiated since 1982 to interrupt transmission and thereby eventually lead to the elimination of leprosy as a public health concern (WHO, 2002).

Although annual diagnosis have significantly decreased after 27 years of MDT programs, leprosy has failed to reach global elimination status with 22,4717 number of patients identified at the beginning of 2007 (WHO, 2007), especially in highly endemic countries including Nepal (Annual Report, 2007). MDT proved highly efficacious in killing the bacteria without inducing resistance, although the optimal length of treatment and associated relapse rates are still controversial (Britton and Lockwood, 2004). A better knowledge of how *M. leprae* is transmitted could benefit strategic drug therapy campaigns and lead to improved control strategies (Truman, *et al.*, 2004).

In order to progress, further epidemiological research of leprosy is necessary, although multiple practical challenges exist. *M. leprae* is the slowest growing bacteria (doubling time of 12-14 days) and cannot be cultured in vitro; thereby, excluding many of the

diagnostic and epidemiological methodologies common to other disease control strategies (Sasaki, *et al.*, 2001). There is a varied spectrum of pathology dependent on the degree and type of host immunological response (Hastings, 1985). In addition, most cases of leprosy are diagnosed in developing countries with significant socioeconomically disadvantaged populations with limited access to adequate healthcare (Moschella, 2004).

Molecular epidemiology is the integration of strain typing molecular techniques with conventional epidemiological approaches to understand the distribution of disease in a population in order to monitor control and surveillance strategies (Small and Moss, 1993, van Embden, *et al.*, 1992). The range of molecular techniques for epidemiological analysis has expanded in recent years and there are now many genotypic methods that enhance our ability to identify the source of infection and time frame of different outbreaks and to allow a high level of discrimination between bacterial strains. Elegant studies on a closely related pathogen, *Mycobacterium tuberculosis*, are providing new insights into the international dissemination dynamics of that disease and bringing new clarity about evolutionary changes in the pathogen among reference strains used in research as well as those isolated over wide geographic areas (Supply, *et al.*, 2000; Supply, *et al.*, 2001; Cole, *et al.*, 2001a). Strain differentiation of *M. leprae* would be of great value for epidemiological investigation to identify the infectious sources of leprosy and to understand transmission patterns of the organisms. In addition, strain differentiation methods for *M. leprae* isolates could be useful in potentially distinguishing relapse from reinfection after the completion of chemotherapy.

Previous molecular studies employing restriction fragment length polymorphism (RFLP) analysis of *M. leprae* isolates using a combination of different enzymes and probes, as well as sequencing of the internal transcribed spacer region of the 16S-23S rRNA operon, have yielded no polymorphic DNA sequences by which to discern potential strains (Williams, *et al.*, 1990a; de Wit and Klatser, 1994; Cole, *et al.*, 2001a). RFLP analysis based on the Southern blot technique is not practical in *M. leprae*, mainly because of the difficulty of obtaining sufficient DNA to run RFLP since *M. leprae* cannot be cultured in vitro. PCR-based molecular typing of *M. leprae* targeting variable numbers of tandem repeats may thus be a more practical option in terms of applicability in clinical laboratories. Some structural variation in the *pol*A (Fsihi and Cole, 1995) and *rpoT* genes

have been described but the value of these elements for differentiating *M. leprae* appears to be limited (Matsuoka, *et al.*, 2000; Chae, *et al.*, 2002; Lavania, *et al.*, 2007).

The *M. leprae* genome has been described as highly degraded and contains a number of dysfunctional pseudogenes (Truman, *et al.*, 2004). In completing the total genomic sequence (Cole, *et al.*, 2001b), several short tandem repeats (STRs) are loosely classified as microsatellies (less than 6bp repeat units spanning a few tens of nucleotides) and minisatellites (6-100 bp units spanning hundreds of base pairs). In some cases minisatellites are defined as variable number of tandem repeats (VNTRs). Dinucleotide, trinucleotide and tetranucleotide repeat polymorphisms discovered shortly thereafter are called STRs. They have the same basic structure as VNTRs but the tandemly repeated consensus sequences are only two to four bases long. The shorter repeat lengths of STR markers make them more compatible with PCR for genetic mapping VNTRs are found at multiple loci. Some loci show substantial variation and are supposed to be an excellent source of polymorphism as the number of repeat units can increase or decrease due to slippage of the strands of DNA during replication by the DNA polymerase (Sreenu, *et al.*, 2007). These markers contain many alleles at each individual locus, not due to the presence or absence of a restriction site but rather, variability in the number of copies of a 15-70 base consensus DNA sequence tandemly repeated within a neighboring pair of restriction sites. The most recent advancement has been in the molecular typing of VNTRs by PCR amplification, multiplex electrophoresis, automated detection and analysis (Supply, *et al.*, 2001). TTC was the first VNTR to be studied and genomic divergence of *M. leprae* by the variation of TTC repeats was observed (Shin, *et al.*, 2000; Matsuoka, *et al.*, 2004). Genotyping according to the TTC repeats for fragments amplified by PCR seemed to be feasible for molecular epidemiological analysis of leprosy transmission. However, the discriminatory power of single VNTR is quite low and the stability of alleles associated with pseudogenes is unclear (Truman, *et al.*, 2004). Hence for better strain differentiation of *M. leprae* for epidemiological studies, it is necessary to include a set of reproducible, stable and discriminating loci (Hunter and Gaston, 1988; Hunter, 1990) potentially including microsatellite and minisatellite loci (Matsuoka, *et al.*, 2004). The use of multiple loci which may differ in polymorphic variability should therefore aid in assessing specimens harboring mixed strains,

determining transmission patterns among endemic populations and providing a means to standardize reference strains of the organism (Groathouse, *et al.*, 2004; Truman, *et al.*, 2004).

Mapping of VNTRs at multiple genomic loci has become a common method for strain identification amongst other bacterial species including *Bacillus anthracis (Keim, et al., 2000)*, *Yersinia pestis* (Klevytska, *et al.*, 2001), *M. tuberculosis* (Spurgiesz, *et al.*, 2003) Since so many answers were unanswered in leprosy regarding its transmission pattern a preliminary study using small sample size was carried out to see the genetic variation in 12 different loci in *M. leprae* genome. Both microsatellite and minisatellite loci were investigated in Nepalese *M. leprae* isolates to determine which polymorphic loci were candidate tools for molecular typing and leprosy epidemiological studies involving leprosy in Nepal. Such development would clearly benefit our understanding of how *M. leprae* is transmitted and could ultimately lead to improved methods to effectively interrupt its transmission and reduce the spread of leprosy.

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CHAPTER-II

OBJECTIVES

General Objective

To investigate the use of mini- and microsatellite molecular typing tools for the assessment of strain variability of *M. leprae* among leprosy patients visiting Anandaban Leprosy Hospital.

Specific Objectives

1. To employ VNTR PCR for a panel of six minisatellite and six microsatellite VNTR loci with *M. leprae* samples isolated from Nepalese leprosy patients and compare findings with previously reported results obtained in Indian and Chinese patient populations.
2. To determine the suitability of using microsatellites and minisatellites as a strain typing tool for *M. leprae*.

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CHAPTER- III

3. LITERATURE REVIEW

3.1 LEPROSY

Leprosy is a non fatal, chronic infectious disease of the skin and peripheral nerves caused by *M. leprae* (Britton and Lockwood, 2004). Autonomic nervous system (Katoch, 1996), liver, kidney and endocrine glands (Martin, *et al.*, 1968; Drutz, *et al.*, 1972; Johny, *et al.*, 1975; Gharpuray, *et al.*, 1977), cardiovascular system and respiratory system (Mitra and Gombar, 2000) are affected as a result of bacillary infiltration due to the type 2 leprosy reaction (Feuth, *et al.*, 2008). Left untreated, leprosy can be a devastating illness because of its potential to cause deformity, especially in the skin, nerves, limbs and eyes (Sasaki, *et al.*, 2001).

3.1.1 Epidemiology of leprosy

Leprosy is an ancient disease that has left behind a terrifying image of mutilation and stigma on the pages of history and still continues to be endemic in 120 different countries including Nepal (Sasaki, *et al.*, 2001). Although effective chemotherapeutic treatment has reduced the numbers of newly diagnosed patients in recent years to approximately 40019 (13.4%), there are an estimated 10-12 million people worldwide currently affected by leprosy (WHO, 2007). The bulk of the disease occurs in south East Asia, Africa, and the Western Pacific (WHO, 1998). During 2007, 254,525 new cases were diagnosed with leprosy worldwide with Brazil, India, Madagascar, Mozambique, Tanzania and Nepal reporting 90% of the cases (WHO, 2008). While Africa has the highest disease prevalence, Asia has the most cases. More than 80% of the world's cases occur in a few countries: India, China, Myanmar, Indonesia, Brazil, Nigeria, Madagascar and Nepal (WHO, 1998). Since the introduction of MDT in 1982, there has been a remarkable improvement in the situation with an enormous effect upon the program of controlling this disease (Araoz, *et al.*, 2006).

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During the past four years, the global number of new cases detected has continued to decrease dramatically by about 20% per year (WHO, 2007). As published in the Weekly Epidemiological Record at the beginning of 2007, the global registered prevalence was 224,717 cases and the number of new cases detected during 2006 was 259,017. During 2006, the number of new cases detected fell globally by more than 40,019 cases (13.4%) when compared with 2005 (WHO, 2007). In some countries, rapid decline in case detection is now visible, e.g. Vietnam, and more recently India which reported 137,685 new cases in 2007, representing a decline of 74% from 537,956 reported in 2000 (Fine, 2008). According to official reports received from 115 countries and territories, the global registered prevalence of leprosy at the beginning of 2006 stood at 219,826 cases out of which south East Asia accounted for about 60% of these cases (WHO, 2006).

Table 1. Global Leprosy situation at the beginning of 2008

WHO Regions	Registered prevalence 01-01-08	New Cases detected 2007	Ratio: Prev/NCD
Africa	30055	31037	0.97
Americas	49388	41978	1.18
South East Africa	120,967	171,552	0.71
Eastern Mediterranean	4240	4091	1.04
Western Pacific	8152	5867	1.39
Totals	212,802	254,525	0.84

Source: WHO, 2008

Table 2. Situation of leprosy in South East Asia at the end of 2006

Countries	Prevalence rate per 10,000	Detection rate per 10,000	Prevalence/Detection ratio
Bangladesh	0.32	4.03	0.79
Bhutan	-	2.47	-
DPR Korea	-	-	-
India	0.72	12.09	0.59
Indonesia	0.97	7.73	1.25
Maldives	0.20	2.67	0.75
Myanmar	-	7.69	-
Nepal	1.43	15.39	0.93
Srilanka	0.72	10.38	0.69
Thailand	0.18	1.05	1.74
Timor-Leste	1.99	22.26	0.90
Total Region	0.68	10.12	0.67

Source: WHO, 2007

Globally, all races appear susceptible to disease; however, it has been reported that Europeans, Chinese and Burmese belong to a 'high susceptibility' group regarding lepromatous leprosy as compared with Africans, Indians and Malaysians (Hastings, *et al.*, 1988). The disease has been reported in all ages ranging from early infancy to the elderly (Kar and Job, 2005). Males have been diagnosed more commonly than females with the ratio of 2:1, which may be due to environmental (clothing habit) or biological factors and delayed presentation of the disease by female patients. The male preponderance in leprosy is not universal since there are several areas, particularly in Africa, where there is either equal occurrence of leprosy in the two sexes or occasionally even a higher prevalence among males by a ratio of about 1.5 to 1 (WHO, 1998). Household contacts, neighbours and social contacts have a greater chance of contracting the disease. Whether this is mainly the result of closer contacts to the index case of the infection, similar genetic and immunological background, environmental factors or a combination of all, is

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not yet resolved (Moet, *et al.*, 2004). In addition poor hygiene, malnutrition, puberty, menopause, lactation of the host is also suspected to play role in the development of the disease (Thangaraj, 1987).

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Nepal, a known leprosy endemic country, is one of the five countries in the world that has not yet achieved elimination defined as a prevalence rate less than 1 per 10000 in the population (Annual Report, 2007). In Nepal, MDT was introduced by the leprosy control program in 1982 and then integrated within the general basic health services in 1998. By 1996, the coverage with MDT had reached all 75 districts of the country. Nepal is committed to the elimination of leprosy as a public health problem and is an active member of the global alliance for elimination of leprosy as a public health problem. Currently, there was 12% reduction in prevalence rate (PR) and 16% reduction in new case detection rate (NCDR). The PR in 42 of the 75 districts is found to be less than one per 10000. The distribution of the disease remains highly focal in Nepal with two regions (EDR and CDR) accounting for about 63% of the cases and more than 80% are detected within the terai region.

Table 3. Trend of leprosy, case detection rate, prevalence rate by region from 2004/2005 to 2006/2007

Geopolitical region	Prevalence Rate			Case Detection Rate		
	2004/2005	2005/2006	2006/2007	2004/2005	2005/2006	2006/2007
EDR	-	1.91	1.56	-	2.62	2.0
CDR	-	1.82	1.58	-	2.21	1.86
WDR	-	1.14	1.05	-	1.27	1.28
MWDR	-	1.39	1.32	-	1.30	1.07
FWDR	-	1.88	1.68	-	1.80	1.59
National	-		1.45	-	1.96	1.65

Source: Annual Report, 2007

3.2 Bacteriology

3.2.1 *M. leprae*, the organism

M. leprae measuring about 2-7 um in length and 0.3-0.4 um in width, are gram positive bacilli that are non -motile, non- spore forming, microaerophilic, pleomorphic, acid fast, slow growing, non tuberculous Mycobacteria. In tissue sections, the lepra bacilli can be seen individually or arranged in clumps resembling cigarette ends with no known toxin production. Analysis of the ribosomal RNA sequences of armadillo grown *M. leprae* by nucleic acid hybridization techniques (Sela, *et al.*, 1989) and by ribosomal RNA sequence comparison (Smida, *et al.*, 1988) revealed that *M. leprae* is most closely related to *Corynebacteria*, *Nocardia* and to mycobacteria like *M. tuberculosis* and *M. bovis*.

M. leprae has never been grown on artificial media but can be maintained in axenic cultures in what appears to be a stable metabolic state for a few weeks (Truman and Krahenbuhl, 2001). As a result, propagation of *M. leprae* has been restricted to animal models, including the armadillo and normal athymic and gene knockout mice (Krahenbuhl and Adams, 2000). *M. leprae* has the smallest genome of all mycobacteria therefore; the capacity for independent growth may be due to extensive loss of genes (Eiglmeier, *et al.*, 2001b). The viability of *M. leprae* harvested from several different sources is now known to vary greatly and many standard laboratory practices, such as incubation at 37ºC, rapidly reduce the viability of this organism (Truman and Krahenbuhl, 2001). While chimpanzees and sooty mangabey monkeys have been shown experimentally capable of developing leprosy, only humans and nine banded armadillos demonstrate naturally occuring leprosy (Truman, 2005).

Unique biochemical characteristics of *M. leprae* reside in the cell wall and associated macromolecules. Basic chemical studies have concluded that the cell wall is a covalently linked peptidoglycan –arabinogalactan –mycolic acid complex similar in composition to all mycobacterial cell walls (Draper, *et al.*, 1987; Daffe, *et al.*, 1993). Some of these molecules are potent immunogens in humans. The dominant lipid in the outer layer of cell wall is PGL-1 which is capable of generating a species specific Ig M antibody response (Cho, *et al.*, 1983).

This pathogen has three specific targets: peripheral neural tissues (SCs) responsible for normal nerve functions, small vessels (endothelial cells and pericytes) and the monocyte-macrophage system (Vejare and Mahadevan, 1987) and where it commonly occur in clumps or globi (Kattan, 2006).

3.3 Transmission

The route of transmission of leprosy remains uncertain and may be multiple, with nasal droplet infection, contact with infected soil and even insect vectors considered the prime candidates (Gelber, 2002). Unlike most other infectious diseases, the mode of transmission of lerposy is still under debate. Initially it was thought that the most likely mode of infection is the droplet infection (Rees and Mc Dougall, 1977) and this seems logical because of the abundance of *M. leprae* in nasal discharge from lepromatous leprosy patients (Brandsma, *et al.*, 2008) hence it is believed to be a plausible mechanism of transmission (Visschedijk, *et al.*, 2000) but some case reports have shown that abraded and damaged skin could be another likely portal of entry hence, the mode of transmission is poorly understood (Brandsma, *et al.*, 2008). Subclinically infected individuals, a vector or environmental contamination are considered as alternative sources of *M. leprae* infection (Argaw, *et al.*, 2006). There is also increasing evidence from nasal PCR that subclinical transmission may exist and that those infected may go through a transient period, not resulting in disease development but allowing transmission of infection to other individuals by nasal excretion (Kampirapap, 2008). Other environmental non human sources like soil (Lavania, *et al.*, 2008), vegetation, water, arthropods (Blake, *et al.*, 1987) and some animal species like the nine banded armadillo (*Dasypus novemcinctus*), chimpanzees, Sooty mangabeys and *Cynomolgus macaques* (Rojas-Espinosa and Lovik, 2001) are considered as other potential reservoir of infection, although definitive laboratory evidence has been difficult to obtain.

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3. 4 Portal of entry and exit of *M. leprae*

Both skin and nasal mucosa contribute to the entry and shedding of *M. leprae* (Job, *et al.*, 2008). Accidental inoculation, penetrating injuries to the unprotected skin, tattooing, vaccination and dog bites can also aid in entry of organisms through skin (Job, *et al.*, 1999). PCR amplification of *M. leprae* in the unbroken skin and nasal secretions of MB patients and their contacts suggested that both skin and nasal epithelia of untreated MB leprosy patients contribute to the shedding of *M. leprae* (Kampirapap, 2008).

3. 5 Genetic factors

Evidences for genetic influences contributing to the susceptibility of developing leprosy are based on: racial differences, familial clustering, pedigree patterns and genetic marker associations (Thangaraj, 1987). Studies have found that Mannan Binding Lectin (MBL_2) gene polymorphism play a role in susceptibility to leprosy and in clinical progression of the disease (de Messias-Reason, *et al*., 2007). Recently Toll like receptor (TLR) and Lymhotoxin alpha (LTA) gene polymorphism were also correlated susceptibility to human disease (Alcais, *et al.*, 2007; Misch and Hawn, 2008).

3.6 *M. leprae* strategies methods for evading destruction by host immune responses

The long generation time of the organism influences the length of a time from infection to diagnosis of clinical disease and the chronic nature of the infection (Fine, 1982). Chronic infection may be a result of suppression of the cellular immune response through induction of suppressor T cells (Bloom and Mehra, 1984; Mehra, *et al.*, 1984) and /or rendering the infected macrophage defective for activation or antigen processing (Desai, *et al*., 1989; Prasad, *et al.*, 1987; Sibley and Krahenbuhl, 1988). This may allow the host to tolerate the large bacterial load seen in lepromatous leprosy patients (up to 10^{14}organisms). In addition, PGL I is also thought to contribute to various biological features like survival of the bacteria intracellulary, as it can scavenge reactive oxygen intermediates (Chan, *et al.*, 1989), resistance to common lysis procedures etc (Brennan and Hunter, 1990).

Three possible strategies for maintaining intracellularism have been proposed. Firstly *M. leprae* bacilli might avoid the bactericidal activities of the phagocytic cells by escaping

from the phagosome and multiplying in the cytoplasm of the infected cells (Mor, 1983). Secondly viable *M. leprae* have been reported to prevent the fusion of phagosomes and lysosomes in murine macrophages (Frehel and Rastogi, 1987; Sibley, *et al.*, 1987), but not in SCs (Steinhoff, *et al.*, 1989). A similar prevention of phagosome –lysosome fusion is an intracellular survival strategy used by *M. tuberculosis* (Hart, 1982). Thirdly inside phagosomes *M. leprae* generates around the bacillus a characteristic electron microscope image called an 'electron transparent zone' (ETZ). The ETZ might represent a physical barrier to prevent degradative or bactericidal proteins of the lysosome from reaching the bacterial surface (Draper and Rees, 1970).

3.7 Clinical forms of leprosy

Infection with the leprosy bacillus can induce diverse clinical features corresponding to the host's immune response (Sasaki, *et al.*, 2001). Clinical lesions range from a small solitary hazy macule to widespread multiple shiny macules. The clinical manifestation of the disease is different in different forms of the disease which are not due to the different strains of *M. leprae* but are the results of the variation in the tissue response of the host to the presence of the leprosy bacilli in the body or host- parasite relationship (Modlin, 1994).

There are mainly two classification schemes: more commonly used WHO classification system and the 5 category Ridley Jopling system (WHO, 1998). Based on the quantity of skin lesions and the numbers of bacilli on skin smear, the two main scategories of WHO classification system are: paucilbacillary (PB) and multibacillary (MB) leprosy. Less than or equal to 5 hypopigmented skin macules and anesthetic patches are resent in PB cases whereas greater than or equal to 5 symmetric skin lesions, nodules, plaques are present in MB cases (WHO, 2000).

The recent and most scientific method which is based on the combination of clinical, bacteriological, immunological and histopathological features of the patient is the Ridley and Jopling classification beginning with early indeterminant (I) leprosy, and continuing with polar tuberculoid (TT), borderline tuberculoid (BT), mid borderline (MB), borderline lepromatous (BL) and lepromatous (LL) leprosy (Ridley and Jopling, 1966).

3.8 Immunology

The host defense events that operate early in infection during the indeterminate phase are perhaps the least understood aspects of the immunology of leprosy (Scollard, *et al.*, 2006). Cell-mediated immunity is strong in tuberculoid patients, yet antibodies dominate responses in lepromatous patients.

Natural versus induced immunity in leprosy

There is a certain degree of natural immunity in leprosy. It is known that some races are more susceptible than others (Thangaraj, 1987). Upon exposure to *M. leprae*, most persons will not develop leprosy. However, it is thought that susceptible individuals develop the indeterminate form of the disease, which may spontaneously heal or progress into the leprosy spectrum. Although little work has been reported on innate immunity and leprosy, it is likely that the nature of the initial inoculum and how well innate immunity responds are instrumental in determining a subsequent path into clinical leprosy (Hagge, *et al.*, 2004). An effective innate immune response in combination with the low virulence of the leprosy bacillus may underlie resistance to the development of clinical disease (Scollard, *et al.*, 2006).

3.8.1 Cell mediated immunity

Experiments have shown that importance of cell-mediated immunity is important in host resistance to leprosy (Hastings, *et al.*, 1988). Cells of the T cell lineage play an essential role in resistance to *M. leprae* (Sibley and Krahenbuhl, 1988) such as MHC restricted CD4 and CD8 cells, CD1 restricted cells. Presence of T helper cells specific for antigen of the leprosy bacillus is a key characteristic of the tuberculoid end of clinical spectrum of leprosy. These T helper cells have been found to be as high as 95% of lymphocytes in tuberculoid granulomas whereas in lepromatous lesions, T cytotoxic /suppressor cells can constitute up to 85% of population (Sehgal, *et al.*, 1989).

When the organisms enter the body, then *M. leprae* antigens accumulate the lymphocytes at those sites principally the cooler parts of body particularly in nerve's SCs. The protective aspects of cell mediated immunity in paucibacillary disease and having high levels of cell mediated immunity possess living organism in their tissues. The collateral

damage to tissues caused by the granulomatous inflammation accompanying cell mediated immunity may have serious, long term consequences such as injury to peripheral nerves. Since it is the only bacteria known to specifically target peripheral nerves (Job, 1971; Ridley, *et al.*, 1987; Stoner, 1979). Various factors may play a role in the loss of nerve function and the generation of crippling deformities.

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3.8.2 Humoral immunity

Because of the intracellular, sequestered location of *M. leprae*, it is doubtful that humoral immunity plays a significant role in resistance to the organism (Hastings, *et al.*, 1988). Although humoral immunity is very effective in fighting many forms of infection, it has little positive effect against *M. leprae* and in fact can cause suffering through the formation of antigen antibody complexes during erythema leprosum nodosum (ENL) reaction. There tends to be an inverse correlation between patients anti *M. leprae* antibody titer and the potency of the patients' cell mediated immune response to the bacillus (Hastings, *et al.*, 1988).

3.8.3 Leprosy reactions

Although immunity in most cases helps the body's defense against the invasion of bacteria and their antigens, there are occasions when the body reacts violently to the antigens (Scollard, *et al.*, 2006). This dynamic nature of the immune response to *M. leprae* leads to spontaneous fluctuations in the clinical state and is termed 'leprosy reaction' (Britton, 1998). Hence it is an acute inflammatory complication often presenting as medical emergencies during the course of treated or untreated Hansen's disease whose cause, mechanism and treatment remain highly problematic (Scollard, *et al.*, 2006). Sometimes the reactions may occur by immunological change following effective chemotherapy, reduction in the bacillary load or may be precipitated by inter current infections (viral, malaria etc), anaemia, mental and physical stress, parturition or surgical interventions. There are 2 types of leprosy: type 1 (reversal reaction, RR) and type 2 reaction (erythema nodosum leprosum, ENL) which appeared to have different underlying immunologic mechanisms (Scollard, *et al.*, 2006).

a. Type 1 reaction

Type 1 or RR, occurs due to a rapid change in the host-parasite relationship (Sansonetti and Lagrange, 1981) usually seen in tuberculoid and borderline forms, but not in patients with polar disease (Scollard, *et al.*, 2006). Skin lesions increase in number, become inflammed and may ulcerate. Peripheral nerves may become tender and swollen and then acute paralysis develops. There may be oedema of the hands and feet.

Reversal reactions are associated with an abrupt increase in cell mediated immune reactions to *M. leprae* antigens (Britton and Lockwood, 2004) occurring before, during or even years after initiation and proliferation (Nery, *et al.*, 2006) of T lymphocytes resulting in a shift towards the tuberculoid pole characterizes upgrading; whereas a downgrading reaction is associated with a decrease in CMI resulting in a shift towards the lepromatous pole. Lesions are localized and bilateral with rapid progressive change which become erythematous (reddish), shiny, slightly raised/ prominent warmer and with the appearance of erysipelas in upgrading reaction whereas in downgrading reaction, lesions worsens and progress towards the lepromatous pole and new skin lesions may appear with much more pain, tenderness when there is nerve involvement (Britton and Lockwood, 2004, Modlin and Rea, 1998).

b. Type 2 reaction (ENL)

Type 2 reaction (Type III humoral hypersensitivity, Coomb and Gel) is likely to result from immune complex formation and deposition (Britton and Lockwood, 2004). It usually occurs in lepromatous or borderline leprosy. There is little change in the leprosy lesions. Crops of tender red lumps appear every 5-7 days which may become vesicula, pustule, bullous, gangrenous and break down with subsequent scarring (erythema nodosum necrotans) occurring anywhere in the body mainly in face, forearm, torso and medial thighs except warmer regions such as scalp, groins, perineum etc (Jacobson and Krahenbuhl, 1999).

3.9 Leprosy diagnosis

The accurate diagnosis of leprosy is of fundamental importance to all aspects of leprosy epidemiology, case management and the prevention of disability. Under diagnosis within a population will allow for the continued transmission and needless individual suffering whereas over- diagnosis will involve over treatment with antibiotics and unnecessary stress and stigma that can lead to misleading epidemiological statistics (Moschella, 2004).

The lack of an in vitro system for the cultivation of *M. leprae* makes positive identification particularly difficult. There is no single diagnostic laboratory test for leprosy and diagnosis remains essentially clinical. Clinical diagnosis of leprosy is dependent upon recognition of disease symptoms and is therefore only possible once the disease has manifested (Duthie, *et al.*, 2007). WHO experts have listed diagnostic criteria as one or more of the following: hypopigmented or reddish skin patches with definite loss of sensation; thickened peripheral nerves; acid-fast bacilli on skin smears/biopsy specimens (WHO, 1998). Other methods have been developed for better diagnosis of leprosy such as inoculation of mouse foot pad, serologic tests like anti PGL 1 antibody test and PCR. Further attempts are being made to develop new tools that will make leprosy diagnosis more simple and reliable (Scollard, *et al.*, 2006). These methods might show promise for epidemiological studies, diagnosis or follow up of treatment. However, current immunological and molecular assays can't be used as diagnostic test as they can not clearly differentiate between infection, contamination or disease (Beers, 1998).

3.9.1 Clinical signs and symptoms

Three cardinal signs remain the basis for the clinical diagnosis of leprosy (WHO, 1998; Hastings, *et al.*, 1988).

- Hypopigmented or reddish skin lesion (s) with definite loss of sensation.
- Involvement of the peripheral nerves as demonstrated by definite thickening with loss of sensation.
- Skin smears positive for acid fast bacilli.

Any one of these signs has been regarded as sufficient for the diagnosis of leprosy and is quite specific in itself hence both sensitivity and specificity are high (Moschella, 2004). But (Saunderson and Groenen, 2000 b) concluded that using one as the diagnostic criteria among the three cardinal signs is inadequate, showing sensitivity of 97%.

Hypopigmented or erythematous macules with definite loss of sensation are present in many newly diagnosed leprosy patients and are often the first clinical sign of the disease. Thickened nerves generally appear later than do skin lesions. The findings of one or more enlarged nerves are more common among MB than PB patients (McDougall, 1996).

3.9.2 Differential diagnosis

Leprosy mimics many dermatological and neurological disorders (Gelber, 1992). The visible signs of a disease may be highly suspected of leprosy but they are never diagnostic by themselves. Included in the differential diagnosis of lesions that resemble leprosy are sarcoidosis, leshmaniasis, lupus vulgaris, lymphoma, syphilis, yaws, granuloma annulare, various other disorders causing hypopigmentation. In lepromatous leprosy, sputum specimens may be loaded with AFB- a finding that can be inappropriately interpreted as representing pulmonary tuberculosis (Gelber, 1992).

3.9.3 Laboratory diagnosis

a. Slit skin smears

Slit skin smears (SSS) are small slit made in pinched skin to avoid bleeding, the edges of which are scrapped; the tissue fluid obtained on the scalpel edge is then smeared on a slide for acid fast staining (AFB). SSS require at least 10^4organisms per gram of tissue for reliable detection; and the microscopic counting of bacilli is scored in units on a bacterial index (BI) of 0-6 (Chae, *et al.*, 2002; Jacobson and Krahenbuhl, 1999). SSS are important in diagnosis, classification, and monitoring response to treatment and control measures in leprosy. The slit skin technique is the WHO preferred method for the detection of early cases of lepromatous leprosy and potential relapse after MDT treatment (Sehgal and Joginder, 1990).

Comment [u9]: Add a simple sentence which describes what a slit skin smear is. Then use another sentence to say that it is the preferred method for WHO.

b. Cultivation of *M. leprae*

There is no satisfactory evidence to support in vitro cultivation of *M. leprae* from the skin or other tissues of leprosy patients. The primary cause of the bacterial uncultiviability in artificial media seems to be the absence of key metabolic and enzymatic pathways that necessitates reliance on host parasitism (Gelber and Rea, 2000). The success achieved in growing the leprosy bacillus in the mouse foot galvanized leprosy research, leading to extensive work in different animal models, development of new chemotherapeutic agents, and the analysis of the biochemical, antigenic and molecular structure of leprosy bacillus (Katoch, 2002). In addition, the nine banded armadillo (*Dasypus novemcinctus*) can be infected with *M. leprae* and has become the main source of organism for immunological and biochemical research including vaccine development. Intravenous inoculation with 10^8-10^9 bacilli regularly results in 10,000 folds increase in the number of *M. leprae* over a span of 18 months and hence is considered as the host of choice for in vivo propagation (Scollard, *et al*., 2006). It is thought that the armadillos hosting ability of *M. leprae* is due to a lower core body temperature of 33-34^{o}C (Truman and Krahenbuhl, 2001).

c. Serology

The single greatest need in leprosy research remains the development of diagnostic tools that can easily detect early infection, and thereby allow intervention before the onset of the neuropathy, disfigurement and disability. Serology is thought to be an affordable tool for this purpose (Parkash, *et al.*, 2006). Many *M. leprae* specific as well as mycobacterial genus specific antibodies in the sera of leprosy patients may indicate current *M. leprae* infection (Cho, *et al.*, 2001) and can be used as marker of bacillary load, prognosis of the disease and predictors of occurrence of future relapse (Jayapal, *et al.*, 2001). The presence of Ig M antibodies to phenolic glycolipid 1 (PGL 1) serves as the basis of a useful test for detecting leprosy infection, particularly the MB form; however, antibodies can persist for considerable time after subsidence of active disease (Araoz, *et al.*, 2006). Although they have some value in population follow up studies, none of these assays has a satisfactory degree of sensitivity and specificity for diagnostic application (Scollard, *et al.*, 2006).

Comment [u10]: missing

d. Histologic findings

The gold standard for the diagnosis of leprosy is a full thickness skin biopsy sample obtained from the advancing margin of an active lesion, fixed in neutral buffered formalin, embedded in paraffin and examined by an experienced pathologist (Scollard, *et al.*, 2006). The primary characteristics to be recognized are histological patterns of the host response in haematoxylin and eosin- stained sections, the involvement of cutaneous nerves, and the identification of acid fast bacilli within nerves using the Fite- Faraco modification of the carbol fuschin (Scollard, *et al.*, 2006).

e. **Molecular methods**

The major advancement in the laboratory diagnosis of Hansen's disease, however, has been the development of methods for the extraction, amplification, and identification of *M. leprae* DNA in clinical specimens using polymerase chain reaction (PCR) and other molecular techniques like direct hybridization with specific probes, isothermal amplification (Bloom, 1994; Iwamoto, *et al.*, 2003; Mukai *et al*., 2006). This is an invaluable addition to laboratory diagnosis and to studies of the basic microbiology of this uncultivable organism, although it is costly and has not yet been approved or become available as a routine clinical test (Scollard, *et al.*, 2006).

Polymerase chain reaction

PCR, one of the most significant advances in DNA based technology, is a rapid, sensitive, inexpensive and simple means of producing relatively large number of copies of DNA molecules from minute quantities of source DNA from viable and non viable cell material even when the cell material is of relatively poor quality (Sambrook, *et al*., 1989). This feature makes the technique extremely useful not only in research but also in commercial uses including genetic research testing, forensics, industrial quality control, and in vitro diagnostics (Bartlett and Stirling, 2003). PCR has several qualitative characteristics such as specificity, efficiency (yield of products), sensitivity and fidelity (error rate). Many infectious agents which are missed by routine culture, serological assays, DNA probes and southern blot hybridization, can be detected by PCR (Sambrook 1989). PCR makes it possible to increase the concentration of a specific gene sequence > 10^6 fold, thereby greatly enhancing the detectability of the target sequence either directly

by agarose gel electrophoresis and polyacrylamide gel electrophoresis or indirectly by hybridization with a labelled complementary DNA probe (Williams, *et al.*, 1990a).

PCR used in diagnosis of leprosy

Several investigators have used DNA amplification through PCR to amplify different gene stretches of *M. leprae*. These include the genes encoding various *M. leprae* proteins 18 kDa (Williams, *et al.*, 1990a), 36 kDa (Hartskeerl, *et al.*, 1989), 65 kDa (Plikaytis, *et al.*, 1990), leprosy serum reactive (Misra, *et al.*, 1995) and rRNA (Cox, *et al.*, 1991), and repetitive sequences (Woods and Cole, 1989). These assays have been reported to be sensitive to 1–10 organisms. These probes and gene amplification assays can be of immense help for the diagnosis of early atypical PB leprosy and also in mass confirmation of diagnosis for epidemiological and research purposes (Katoch, 2002). However, because of the persistence of weak signals in some cases a long time after effective treatment (Katoch, 1999), DNA-based PCR assays appear to have limited application in monitoring treatment, particularly in distinguishing late reactions and relapses (Katoch, 2002). Techniques such as reverse transcription PCR targeting 16S rRNA (Kurabachew, *et al.*, 1998) and nucleic acid sequence-based amplification targeting 16S rRNA have been reported to be useful for the determination of viability of *M. leprae* (van der Vliet, *et al.*, 1996).

3.10 Molecular epidemiology

Molecular epidemiology is the integration of molecular techniques to track specific strains of pathogens with conventional epidemiological approaches to understand the distribution of disease in population (Small and Moss, 1993; van Embden, *et al.*, 1992). Molecular epidemiology attempts to utilize a multidisciplinary approach to identify factors that determine disease causation, propagation/ dissemination and distribution (in time and space) which are primarily achieved by associating epidemiologic characteristics with the biologic properties of clinical isolates (Mathema, *et al.*, 2006). These studies can give insight into the international dissemination and dynamics and permit analysis of evolutionary changes within a pathogenic population (Supply, *et al.*, 2001) assuming that patients infected with strains of identical types are epidemiologically linked, while those infected with strains of different types are not (Savine, *et al.*, 2002).

Hypervariable markers blur epidemiological links and lead to their underestimation, whereas markers evolving too slowly erroneously link distantly related isolates, leading to overestimation of transmission rates (Savine, *et al.*, 2002). This molecular typing is particularly important in the study of chronic disease such as leprosy where patients with recurrent leprosy can be chronically infected with a given strain and relapse due to reactivation of that strain or in contrast can be reinfected by a different strain after cure.

Molecular techniques available for strain typing

Molecular typing methods available in recent times that help to identify emerging pathogenic strains or clones within species are RFLP, spoligotyping, Pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism, ribotyping, plasmid typing, single nucleotide polymorphism typing etc.

3.10.1 Genome of *M. leprae*

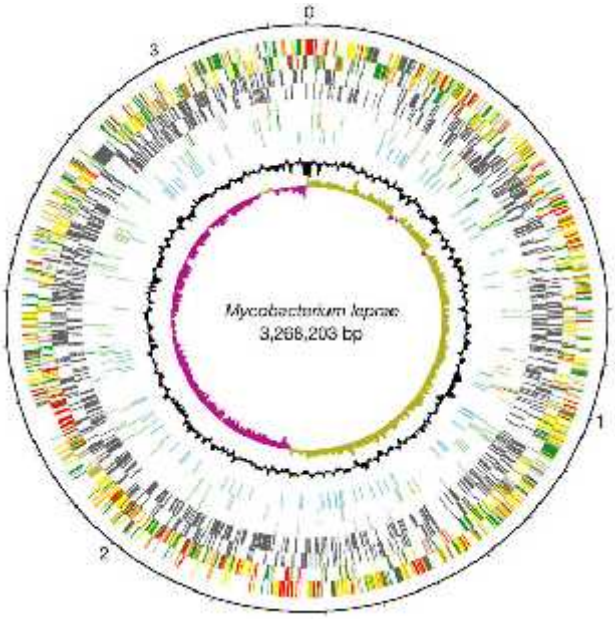

Figure 1**: Organization of genome of TN strain of *M leprae* showing the position and orientation of known genes, pseudogenes and repetitive sequences** (Cole, *et al.*, 2001b). From the outside: circles 1 and 2 (clockwise and anticlockwise) genes on the - and + strands, respectively; circles 3 and 4, pseudogenes; 5 and 6, *M. leprae* specific genes; 7, repeat sequences; 8, G+C content; 9, G/C bias (G+C)/. Genes are colour coded using the following functional categories[8]: 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); cell wall and cell processes (green); PE and PPE protein families (magenta); virulence, detoxification, adaptation (brown).

Comparison of the recently sequenced genome of *M. leprae* with other mycobacterial genomes revealed that 49.5% of the genome is occupied by protein coding genes (Figure 1). Of 1,604 potentially active genes, 1439 are common to both *M. leprae* and *M.*

tuberculosis. Twenty seven percent of the genome (~ 1100) is occupied by recognizable pseudogenes which are inactivated, non -functional regions in the genome that have been rendered inessential and have arisen as a consequence of accumulating mutations and by the loss of different sets of sigma factors at different time points during the course of evolution (Madan Babu, 2003).The remaining 27.5% is non-coding that probably contains gene remnants mutated beyond recognition together with the regulatory sequences that usually occurs in the intergenic regions (Eiglmeier, *et al.*, 2001a).

Comment [u11]: What is this ref??

Table 4. Comparision of the genome of *M. leprae* with that of *M. tuberculosis*.

Comparison of genome features		
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.)	1,604	3,959
Pseudogenes (no.)	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

Source: Cole, *et al.*, 2001a

M. leprae genome downsizing

Comparison of the genome sequence of the armadillo-passaged strain of *M. leprae* from Tamil Nadu, India known as the TN strain with that of the close relative *M. tuberculosis* led to a major breakthrough (Monot, *et al.*, 2005) revealing a drastic gene reduction and decay in *M. leprae* affecting many metabolic areas, exemplified by the retention of a minimal set of genes required for cell-wall biosynthesis (Vissa and Brennan, 2001). Topologically equivalent, the genome of *M. tuberculosis* and *M. leprae* were roughly 4.4 Mb in size but due to the extensive genome downsizing *M. leprae* genome size has become 75% less than that of *M. tuberculosis H37Rv* (MTH) genome (Cole, *et al.*, 1998; Cole, *et al.*, 2001b; Eiglmeier, *et al.*, 2001b) and resulted in 1.1 Mb reduction in genome size that would have eliminated ~1100 protein coding genes and therefore it produces 3000 proteins (Eiglmeier, *et al.*, 2001a). Moreover it has been found that the genome has resulted from recombination between dispersed copies of the repetitive elements resulting in removal of entire metabolic pathways, regulatory genes and accumulation of more than 1130 pseudogenes (Monot, *et al.*, 2005) which makes the organism dependent on host metabolic products (Britton and Lockwood, 2004).

Repetitive DNA

`Non-coding' DNA generally contains DNA sequences for which no function has yet been established or possibly for which no function exists. Such sequences may either be single copy (spacer DNA between the coding regions of the genome) or exist in multiple copies, thus being called repetitive DNA (Fowler, *et al.*, 1988). Repetitive DNA which are supposed to be the prominent constituents of genomes in higher eukaryotes, consists of simple homopolymeric tracts of a single nucleotide type [Poly(A), Poly(G), poly(T) or Poly(C)] or of a small numbers of several multimeric classes of repeats built from identical units (homogenous repeats), mixed units (heterogeneous repeats) or degenerate repeat sequence motifs. Approximately 2% of the *M. leprae* genome is composed of repetitive DNA. The genome contains a few repeating elements and limited variation between different isolates has already been reported for some of them. Recently a few loci for allelic diversity have been identified including small insertion sequences and tandem repeating elements (Cole, *et al.*, 2001a).

Interspersed genome-wide repeats

Interspersed repeats are mobile genetic elements often referred to as insertion sequences (IS) present in several copies and are said to occur by transposition. Also known as composite transposon, IS are important component of bacterial genomes and their transposable ability has mutational potential in coding or regulatory regions (Mahillon and Chandler, 1998). There are more than 26 IS elements together with four families of dispersed repeats present in five copies or more: *RLEP* (37 copies), *REPLEP* (15 copies) *LEPREP* (8 copies) (Cole, *et al.*, 2001a). The known IS elements in the *M. leprae* genome are *REP13* element (1051bp) with 65% similarity with IS 1081 of *M. tuberculosis* (Poulet and Cole, 1995) and other known direct repeats are *REP 9* (309 bp), *REP 14* (52 bp) (Smith, *et al.*, 1997).

Tandem repeat (TR)

Tandemly repeated DNA, also known as satellite DNA, been detected in both prokaryotes and eukaryotes (Shelenkov, *et al.*, 2006). Tandem repeats (TRs) in DNA are two or more contiguous, approximate copies of a pattern of nucleotides of sizes 2-80 bp (Lupski, *et al.*, 1996). 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. Over time, individual copies within a TR may undergo additional uncoordinated mutation so that typically only approximate tandem copies are present (Benson, 1999). The STR regions in *M. leprae* that showed genetic diversity are reminiscent of contingency loci in other bacterial genera exlopiting copy number variations that occur due to slipped strand mispairing (Young, 2003). In the *M. leprae* genome, there are various tandem repeats and some of them studied extensively are: TTC, *RLEP*, *rpoT* and various other microsatellites and minisatellites that showed extensive variation suggesting a level of diversity suitable for local molecular epidemiology (Shin, *et al.*, 2000; Matsuoka, *et al.*, 2005; Zhang, *et al.*, 2005; Groathouse, *et al.*, 2004).

Short tandem repeat (STR)

A short tandem repeat (STR), also classified as microsatellites and minisatellites, is a short sequence in DNA normally of 2-10 bp runs of primarily mono, di, tri and tetranucletide repeats repeated 10-20 times within the non coding intron region (Shelenkov, *et al.*, 2006). Short tandem repeat polymorphisms (STRPs) are classified by the different number of copies of the repeat element that can occur within a population of individuals. STRPs offer several advantages in mapping over other traditional methods like RFLP, spoligotyping, etc. One of the unique properties of the SSRs is their high degree of polymorphism by virtue of variability in their repeat number at most loci. Most repeat mutation rates are about 10 to 10^4 fold higher than those of non repeated regions and lie between 10^3 and 10^6 per cellular generation (Legendre, *et al.*, 2007). In certain cases, mutation rates also vary greatly from repeat to repeat and as a consequence, not all tandem repeats are suitable genetic markers.

Microsatellite

Microsatellites or simple sequence repeats (SSRs) consist of only 1 to 6 bp scattered through the genome. Regions with high occurrence of microsatellites are called contingency loci (Moxon, *et al.*, 1994). These SSRs are susceptible to replication slippage, slipped strand mispairing, gene deletion, gene substitution, or gene addition resulting in hypervariable length so there is increase or a decrease in the copy number of the repeat element during cell division. Microsatellites have been extensively used for mapping of genomes of higher eukaryotes (Sreenu, *et al.*, 2007). Although the nucleotide sequences of microsatellite repeats vary frequently, they have been shown to be a rich source of highly polymorphic genetic markers and serve as excellent molecular markers for genotyping, strain differentiation and epidemiological analysis in humans and other mammals. It is particularly suitable for population studies because STRPs present high heterozygosities and genetic diversities. These can be relatively easily characterized using a combination PCR amplification of each locus followed by electrophoresis to separate alleles. However, it has been suggested to use this method only if the organism lacks other types of genetic variability (Hughes and Queller, 1993), because amplicons

will be difficult to size accurately for bacterial typing (Chang, *et al.*, 2007). For leprosy, the initial studies demonstrated very little genetic diversity among *M. leprae* isolates (Matsuoka, *et al.*, 2004).

Mutations in microsatellites are believed to be dependent on their tract lengths and that the long tracts of more than seven repeats are more prone to slippage than shorter tracts and hence any such tract can be called as potential polymorphic microsatellites (PPMs) which are the mononucleotide tracts. *M. leprae* is relatively rich in PPMs of which 74% are found in non coding regions whereas 80-90% of the PPMs in *M. avium*, *M. bovis*, *M. tuberculosis* DC 1551 and MTH are in the coding regions (Sreenu, *et al.*, 2007). Five out of the thirty-eight PPMs in clinical isolates of *M. leprae* have already been tested and reported to be polymorphic (Groathouse, *et al.*, 2004).

Comment [u12]: What are these?

Various VNTRs in *M. leprae* genome were studied earlier and found to be potentially useful as genetic markers for strain typing (Truman, *et al.*, 2004; Young, *et al.*, 2004; Weng, *et al.*, 2007). Microsatellites like AT, TA, GGT, TTC, GTA, AC are under study. Different copy numbers in unrelated isolates can potentially be used as possible genetic markers for molecular typing of the organisms (Truman, *et al.*, 2004; Young, *et al.*, 2004).

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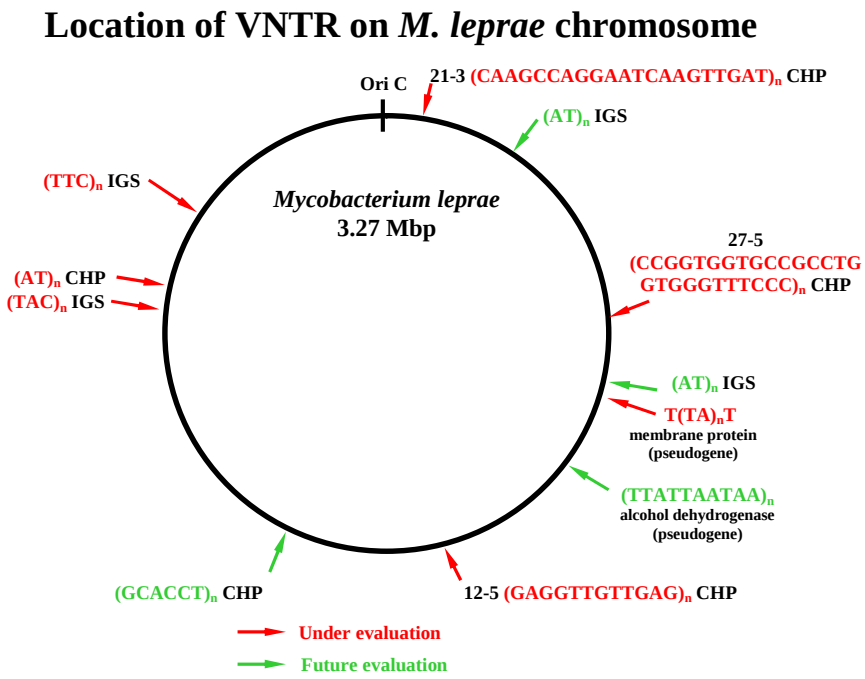

Figure 2: **Variable number of tandem repeats (VNTR) within the *M. leprae* genome**. The VNTRs under study are marked with red arrow and the VNTRs for future study are marked with green arrow. Green or red colored alphabets are microsatellites.Within the parentheses are given the sequence of that particular locus (Truman, *et al*., 2004).

Comment [TLM13]: Isn't this in ne of their papers? If so, it needs to be cited properly and the figure legend needs to be more explanatory. This figure may be better before the loci descriptions.

Variable number of tandem repeats (VNTR)

Ubiquitous in eukaryotes and human DNA, VNTR minisatellites are short nucleotide sequences ranging from 14 to 100 nucleotides long repeated 4-40 times (Shelenkov, *et al.*, 2006). Nakamura, *et al. in* 1988 introduced the terminology VNTR which represent a single locus showing inter-individual length variability (Jeffreys, *et al.*, 1985). It describes allelic variants of tandemly repeated core sequences which represent simple head to tail repeated sequences organized into clusters scattered on chromosome and are highly polymorphic. A number of repeats at a given locus can display high variability and diversity; they can have great discriminatory capacity (Vitorino, *et al.*, 2005). Individual strains within a bacterial species often maintain the same VNTR sequence element but with different copy numbers (Bzymek and Lovett, 2001). These are often caused by slipped strand mispairing that may occur in combination with inadequate DNA mismatch repair pathways during DNA replication (Strand, *et al.*, 1993).

Typically, VNTR molecular subtyping system consists of a series of VNTR loci around which PCR primers are designed. The resulting amplicons are then separated by electrophoresis. Differences in amplicon size at individual loci are assumed to be due to variation in repeat copy number at that locus and the banding or peak patterns are scored accordingly. VNTR-PCR amplification involves amplifying the DNA using PCR primers in the sequences flanking this region to detectable levels. The number of TR units can be determined from the size of the PCR product using various methods (including automated fragment analysis, agarose gel electrophoresis, AGE and polyacrylamide gel electrophoresis, PAGE). DNA fragment analysis is the state of choice for current global studies. The use of different fluorescent dyes and amplicon size ranges allows multiple loci to be multiplexed and subsequently analyzed by DNA fragment analysis. It has been shown that VNTR can demonstrate genetic variation more specifically than that detected by traditional serological and biochemical genetic markers (Papiha, *et al.*, 1996), since the PCR assay using the regions flanking the repeats are generally well-conserved targets for primer design (van Belkum, *et al.*, 1998). This is the reason that they have been extensively studied for paternity and genetic linkage test between human and individuals (Jeffreys, *et al.*, 1985).

3.11 Methods used previously for *M. leprae* strain typing

Elegant studies on a closely related pathogen, *M. tuberculosis*, are providing new insights into the international dissemination dynamics of that disease and bringing new clarity about evolutionary changes in the pathogen over wide geographic areas or among reference strains used in research (Supply, *et al.*, 2000; Supply, *et al.*, 2001). Unfortunately, no definitive methods for epidemiological analysis have been developed to subtype global *M. leprae* isolates by genomic divergence to better understand the mode of transmission leprosy although VNTRs are rapidly assuming this position (Matsuoka, *et al.*, 2005).

In previous studies restriction fragment length polymorphism (RFLP) analysis of *M. leprae* was unable to identify differences among *M. leprae* isolates from geographically different areas (Williams, *et al.*, 1990b; Rastogi, *et al.*, 1999). Various trials to type *M. leprae* with RFLP based on constant patterns of restriction fragments and results from DNA probing (Williams, *et al.*, 1990a; Rastogi, *et al.*, 1999; Clark-Curtiss and Walsh, 1989) did not show genetic diversity in geographically distinct isolates of *M. leprae* Further, single-strand conformation polymorphism (SSCP) of DNA sequences encoding 16S and 23S rRNA were identical in *M. leprae* isolated from different multibacillary leprosy patients (de Wit and Klatser, 1994). A recent report on *M. leprae*-specific repetitive sequences, *RLEP*, suggested variability among *M. leprae* isolates, since PCR amplification of this repeat showed different intensities and the *RLEP* sequence was absent in certain *M. leprae* isolates (Fsihi and Cole, 1995).

Comment [TLM14]: It will be best to discuss in one or two concise paragraphs the various methods that have been used to attempt strain typing with ML contrasting their limitations. Thorough descriptions of methods is unnecessary.

The *rpoB* gene that encodes the ß subunit of RNA polymerase contains a highly polymorphic region in which DNA sequences can be used for species identification (Lee, *et al.*, 2003). The gene seems to tolerate a more diverse sequence alteration without causing any changes in protein function. The tolerable sequence variation in the *rpoB* gene becomes a useful clue for species identification of mycobacteria. Multiple mutations were reported in the *rpoB* gene resulting in rifampicin resistance and this mutation can be detected by rapid novel multiple primer PCR amplification refractory mutation system

(MARS) (Sapkota, *et al.*, 2008). The utility of these mutations in strain identification has not yet attempted (Sreenu, *et al.*, 2003).

Some workers have investigated single nucleotide polymorphism that is useful in studies of long range transmission of leprosy. Three SNP were found to be informative and have been used to reconstitute the evolution and global spread of *M. leprae* (Monot, *et al.*, 2005). Shabana, *et al.* (2003) reported SNP at the 154 th bp position within *M. leprae's* 18kDa heat shock protein and concluded the polymorphism may be used as a molecular marker in strain typing of *M. leprae* within endemic populations. Although SNP analysis is technically easier than VNTR analysis, as only interrogation of single position is a required, later study failed to detect any differences between *M. leprae* isolates using the technique (Monot, *et al.*, 2008).

With deciphering *M. leprae* genome in 2001 repetitive sequences known as TRs are used as genetic markers for strain typing. The *rpoT* gene (1725 bp) encoding the Mycobacterial principle sigma factor of RNA polymerase (also known as sig A) having either three or four copies of a six base tandem repeats was used for genotyping of *M. leprae* isolates (Matsuoka, *et al.*, 2005). Several attempts to type *M. leprae* globally concluded that the gene was suitable for genotyping facilitating the epidemiological and anthropological analysis of the origin and spread of leprosy (Matsuoka, *et al.*, 2000; Chae, *et al.*, 2002; Matsuoka, *et al.*, 2005; Srisungngam, *et al.*, 2007; Lavania, *et al.*, 2007).

After sequencing of the *M. leprae* genome, various scientists identified several VNTRs and other elements that could prove useful for discriminating *M. leprae* strains from patients in various geographical areas (Cole, *et al.*, 2001a; Shin, *et al.*, 2000; Matsuoka, *et al.*, 2004; Truman, *et al.*, 2004; Srisungngam, *et al.*, 2007; Monot, *et al.*, 2008). However, the discriminatory power of a single VNTR is quite low, and the stability of alleles associated with "pseudogenes" is unclear (Truman, *et al.*, 2004).

3.12 Multiple locus VNTR analysis (MLVA)

The analysis of set of VNTR loci is known as MLVA sub typing systems (Groathouse, *et al.*, 2004). MLVA was proposed as a suitable method for strain typing of *M. leprae* for molecular epidemiological studies (Groathouse, *et al.*, 2004; Young, *et al.*, 2004; Truman, *et al.*, 2004; Zhang, *et al.*, 2005; Weng, *et al.*, 2007; Young, *et al.*, 2008). It has provided unprecedented differentiation among strains of bacterial species previously thought to have very little sequence variation otherwise providing useful genetic discrimination whether the populations were worldwide or regional or from a localized outbreak (Keim, *et al.*, 2000; Noller, *et al*., 2003). MLVA typing method similar to the MIRU-VNTR method for *M. tuberculosis* (Roring, *et al.*, 2004) capitalizes on the inherent variability encountered in many regions of repetitive DNA that display hypervariability, enabling their exploitation for strain typing (Keim, *et al.*, 2000; van Belkum, *et al.*, 1998). It has been compared with other traditional methods such as RFLP, spoligotyping, MLST and is found to be suitable based on discriminatory potential and convenience in laboratory applications (Weng, *et al.*, 2007).

3.13 VNTR as a tool for genotyping of *M. leprae*

Strain typing by genomic polymorphism might be of great value in efforts to: understand the mode of transmission; monitor outbreak; distinguish between reactivation and reinfection as a cause of disease; study mycobacterial population biology from local and global perspectives and estimate the extent of recent transmission (Young, *et al.*, 2004). Although VNTR sequence analysis has emerged as a valuable tool for the genotyping of several bacterial species, the major drawback is that the evolution of repetitive DNA may be too rapid thereby compromising epidemiologic concordance (Kimura *et al*., 2009).

A panel of VNTR markers has been found to be suitable for molecular epidemiological studies of various bacterial pathogens including: *M. tuberculosis* complex (*M. bovis)* (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). The understanding of the epidemiology of tuberculosis has advanced significantly with the availability of tools for screening the genetic diversity by VNTRs and has demonstrated discrimination between

outbreaks strains (Mazars, *et al.*, 2001; Supply, *et al.*, 2000). By analogy with *M. tuberculosis*, PCR-based typing systems for polymorphic loci could be applied to leprosy cases at the multibacillary end of the disease spectrum.

VNTR has been examined as potential typing markers and found to vary in copy number between strains of *M. leprae* thus arousing interest in their application as epidemiological tools (Matsuoka, *et al.*, 2000; Shin, *et al.*, 2000; Groathouse, *et al.*, 2004; Truman *et al.*, 2004; Monot, *et al.*, 2005). Initial studies for leprosy, however demonstrated very little evidence of any genetic diversity among *M. leprae* isolates (Cole, *et al.*, 2001a; Clark-Curtiss and Walsh, 1989). Single VNTR TTC was initially used for typing *M. leprae* turned out not to be sufficiently discriminatory (Shin, *et al.*, 2000; Matsuoka, *et al.*, 2004; Truman, *et al.*, 2004; Pandey, 2005; Srisungngam, *et al.*, 2007; Monot, *et al.*, 2000). More-extensive sets of VNTR loci have been described subsequently (Groathouse, *et al.*, 2004; Zhang, *et al.*, 2005; Weng, *et al.*, 2007) including a system based on a combination panel of minisatellite and microsatellites loci which appear more appropriate for monitoring the spread of *M. leprae* over shorter epidemiological distances such as within a region or a large city owing to their more dynamic nature (Monot, *et al.*, 2008). This method relies on PCR amplification using primers specific for the flanking region of the VNTR and on determination of the sizes of the amplicons which reflect the numbers of the amplified VNTR copies. Their use provides typing data in a portable numerical format, adequate for studying the global molecular epidemiology of infectious agents (Frothingham and Meeker-O'Connell, 1998).

A panel of 11 polymorphic loci identified within a small set of four isolates of armadillo derived *M. leprae* were screened namely: 6-7, 12-5, 21-3, 27-5, C20, AT17, TA18, GTA9, AGA20, TTC and *rpoT* (Groathouse, *et al.*, 2004). The study demonstrated the presence of at least two alleles for all minisatellites, further confirmed by sequencing. Microsatellite sequences AT17, C20, TA18 and GTA9 showed polymorphism; but due to the small size of the repeat unit, it was necessary to sequence the products to properly analyze the results. Groathouse, *et al*. (2004) screened for VNTRs within minisatellites and microsatellite loci in the same set. While no polymorphism was observed in AC9 sequence by PCR and electrophoresis, sequencing revealed two alleles different within the sample population: 8 and 9. AT17 and AC 8b were found to be highly polymorphic.

Among trinucleotide microsatellites, only GGT 5 was found to be polymorphic. Likewise out of 6-3b, 7-3, 10-4, 15-3 and 23-3 minisatellites, only 23-3 was found to be polymorphic.

Researches have shown that four microsatellites, namely: GAA21, TA18, AT17, C16, G8 and GTA9 are polymorphic. GTA 9 demonstrated the smallest number of different alleles while TA18 demonstrated a wide range of alleles. C16G8 failed to yield reproduceible results. As no particular genotype was seen to be associated with different regions or hosts of origin, additional loci were recommended for the development of a more robust typing system (Truman, *et al.*, 2004).

One of the recent study compared microsatellites TTC, AGT and AT 17 profiles for sample taken from different anatomic sites from the same individual and demonstrated that identical microsatellite profiles could differ at one or more loci for *M. leprae*. These results suggest either the presence of heterogenous population or that the bacterium might have undergone mutation within the host (Young, *et al.*, 2004).

During investigation of 44 STR loci of *M. leprae* TN genome sequence including 33 microsatellites and 11 minisatellites, 33 were found to be polymorphic (Zhang, *et al.*, 2005). Among 32, 9 STRs were suitable for identifying genotypes according to the discriminatory capacity, stability and reproducibility. Identical repeats from different skin lesions on same individual was obtained and rarely mismatch profile was seen at TTC locus which probably suggested bacterial population co existed instead of resulting from replication slippage.

VNTR analysis of eleven loci within *M. leprae* genome obtained from Chinese biopsy samples were performed and found that gene *rpoT* and loci GGT-5, AC 8b, 12-5, 21-3, 27-5, 18-8 and 23-3 were not sufficiently polymorphic enough to be used for strain typing within their sample population (Weng, *et al*., 2006); however loci GTA-9, AC-9 and 6-7 showed significant allelic diversity between isolates. Hence further analysis in the Younnan Province of China were performed using microsatellites AC9, AC8a, GAA21, GTA9, AT17, AT15, TA18 and minisatellite locus 6-7 selected on the basis of their allelic diversity for strain differentiation. This method of MLVA strain typing identified several clusters of patients whose *M. leprae* specimens shared similar VNTR

profiles. Study on multicase families revealed closely matched intrafamilial VNTR profiles in six multicase families. Moreover VNTR pattern related to those found in some multicase families were also detected in the same or adjacent townships indicating the utility of VNTR strain typing to identify and detect short range transmission events (Weng, *et al.*, 2007).

Studies have shown that VNTR pattern of *M. leprae* from different anatomic sites were same but isolates from nerves and skin showed significantly different VNTR patterns (Young, *et al.*, 2008; Monot, *et al.*, 2008). They concluded that VNTR could be too dynamic, but may provide insights into the development of disease within individuals and for tracking short range transmission. Hence the result hinted that it may not provide a robust tool for distinguishing between relapse and reinfection from reactivation as genetic divergence is particularly likely between bacilli inhabiting different tissues (e.g. skin, nerve) (Monot, *et al.*, 2008).

The first molecular epidemiological study of Nepalese *M. leprae* isolates using TTC loci as the genetic marker was performed which showed wide variation range (Pandey, 2005). A necessity of strain typing using a panel of VNTRs including both microsatellites and minisatellites was felt so the present study was proposed to carry out strain typing of Nepalese *M. leprae* isolates 12 different VNTRs including both minisatellite and microsatellite. Single nucleotide differences are more difficult to detect by PCR and require sequencing hence it is not practically feasible in developing countries like Nepal. So PCR amplicons were analysed by 8% PAGE and VNTR data analysis by web based programme.This epidemiological study may help in validation of the VNTR loci in *M. leprae* genome that could be potentially used in strain variation in Nepal.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials

A complete set of reagents, chemicals, equipments, glasswares and plasticwares used for this study is given in Appendix I.

4.2 Methodology

4.2.1 Study site

The study was carried out from May 2007 to July 2008 at the Mycobacterial Research Laboratory (MRL), Anandaban Hospital (AH), Tikabhairab Lele, Laliitpur, Nepal in collaboration with Colorado State University (CSU), Fort Collins, USA.

4.2.2 Study sample

A total of 52 punch or elliptical skin biopsies were obtained from AH-MRL Skin biopsy repository containing left over samples after mouse foot pad (MFP) inoculation for clinical testing. Detailed information of the patient regarding name, age, sex, ethnic group, location (zone, district, development region), BI, household contacts, stage of reaction was taken. All human biopsies were obtained in accordance with a defined protocol followed at MRL after taking informed patient consent.

4.2.3 Sample collection and processing

Normally elliptical or punch biopsy (3x6mm) is taken from nodules or lesions with local anesthesia of a new or reinfected patient with BI≥2. The biopsy taken was divided for histopathology and MFP inoculation. Further biopsy processing was done at MRL MFP lab under sterile condition within a laminar flow biosafety cabinet. Biopsy samples were bissected in a sterile petri dish and macerated with the help of sterile curved scissors. A direct smear was prepared from the sample for the approximate estimation of BI. Tissues were then ground in a 15 ml tissue grinder for a few seconds after adding a few drops of 0.1%BSA (Sigma, USA) in normal saline (2ml) and removing fat and epidermis. The suspension was then recovered and appropriate dilution was made on the basis of BI. Final AFB/ml was determined by Ziehel-Neilson (ZN) staining of the slide prepared from

diluted samples. Further dilution of the suspension was prepared as standard innoculum ($1x10^4$bacteria/ml) for injection of MFP with the remaining fine suspension stored at -20ºC for VNTR study.

4.2.4 DNA extraction

A portion of each minced biopsy samples was processed for Freeze and Boil method to extract genomic DNA (Woods and Cole, 1989). Briefly, the minced biopsy was immersed in liquid nitrogen (-196ºC) for 1 min and then immediately heated at 100ºC for 1 min in dry heat block (DB2A Techne, UK). Finally crude DNA suspension was obtained after alternate repetitition of cycle for 5 times. DNA quantification was then performed by spectrophotometer (8625 UNICAM, UK) (Sambrook, *et al*., 1989).

4.2.5 VNTR identification and selection of primer

VNTR sequences within genome of the TN strain were previously identified using the 'Search Pattern Programme' within the Leproma website (Jones, *et al.*, 2001), the Tandem Repeats Finder (Benson, 1999) and the MIC db 1.0 (Sreenu, *et al.*, 2003) for simple nucleotide repeats. The primer sets were identified by using Oligo 6 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, Co, USA). Primers used in the study were kindly provided by Dr. Vara Vissa of Colorado State University, USA. Primer sequence information and amplicon length are provided in Table 5.

Table 5. Primers used in the PCR amplification of VNTRs (Jones, *et al.*, 2001)

Loci	Primer Sequence (5' to 3')	Melting Temp °C	Repeat unit (bp)	Expected Amplicon length (bp)
21-3	**FP**:GAATCTGACCTTTCGGAAATG **RP**:CGATGCAGCTTCCTACGG	60 58	21	312
12-5	**FP**:CTGGTCCACTTGCGGTACGA **RP**:GGAGAAGGAGGCCGAATACA	62 62	12	289
23-3	**FP**:AACACGCTCCGATTCCGCTAC **RP**:GCCGTAAATCCGCTCCC	66 56	23	326
27-5	**FP**:ATTGAGCAGATGGCCGGTC **RP**:AGCAGTCGGCACGCCCTT	60 60	27	327
6-7	**FP**:GCCATCGTTGTCGGTTCATC **RP**:CGGAGGAGGTGGGTACGGT	62 64	6	268
18-8	**FP**:GCCCGTCTATCCGCATCAA **RP**:GCAAAGATCAGCACGCCAAT	60 60	18	348
GTA9	**FP**:AGCCTTAGTCGCGCAGATG **RP:**TCCGCTGTCCGTCCGCTGA	60 64	3	307
GGT5	**FP**:GCAGCGGTGTAACAGCATACG **RP**:TGTCTGCCTTGCGAAACGGTC	66 66	3	242
AC9	**FP**:GACTGTAAAGTTCAGCCGAGG **RP**:GACTGGATGTCGGCACCC	64 60	2	238
AC8b	**FP**:GCCCACTTACCTCAACCAAC **RP**:CCTATAACGGCACTCAGTCCA	62 64	2	390
AT17	**FP**:TCTCCAACATGCTGCGACA **RP**:GTACAGCGGCCTGATCGAA	60 60	2	181
TA18	**FP**:CGTGCGTCGTGTGTAGGC **RP**:GACGTGGCAACATCGAAGTT	62 60	2	230

FP=Forward primer, RP=Reverse primer

Optimization of VNTR PCR

The most important threat to PCR is contamination. A conventional three room approach was followed to avoid PCR contamination: the clean, grey and dirty area.The clean room was used for PCR master mixture preparation, grey area for template DNA addition and dirty area was used for the amplification and analysis of PCR product (Parish and Stoker, 1998). A laminar flow cabinet with a UV lamp (BH12 Labcaire system Ltd, UK) was used as work station after cleaning with 20 % sodium hypochlorite (Sigma, USA) to destroy any extraneous DNA before PCR setup.

4.2.6 PCR amplification of VNTRs

The recommended DNA concentration for PCR is approximately 10ng/ml; but in this study, different concentrations of DNA (1ng, 2ng, 5ng and 10ng) were prepared for optimization. The PCR mixture consisted of Taq polymerase, dNTPs and buffer (Bangalore Genei, BG), 10nM of each primer and 1ng, 2ng, 5ng or 10ng of *M. leprae* genomic DNA. Optimization of cycle parameters was carried out by adjusting the annealing time, temperature and denaturation time from the recommended cycle parameters by CSU to yield bright, discrete bands in PAGE. Following an enzyme activation step at 95º C for 5 mins, 40 cycles of standard PCR were performed using the model T3 thermocycler (T3 Whatman; Biometra, Germany) in which denaturation was done at 95º C for 15 or 20s, annealing for 15 or 20s at different annealing temperatures for each primers and 1 min of extension at 72º C with a final extension step of 5 mins at 72 ºC prior to storage at 4º C.

Table 6. Optimized PCR components for VNTR PCR

Components	Working concentration	Volume taken(ul)	Final concentration/ 25µl rxn
Sigma Water	-	7.7	-
dNTPs	1mM	5	200µm
PCR buffer	10x	2.5	1x
Primer A	10µm	1	10µm
Primer B	10µm	1	10µm
Mgcl2	2.5mm	2.5	2.5µm
Taq polymerase	3U/µl	0.3	1U/µl
Template DNA	1-10ng/ml	5	-
Total		25µl	

Table 7. Optimized Cycle Parameters within thermocycler

VNTR loci	Denaturation Time (sec)	Annealing	
		Time (sec)	Temp (ºC)
21-3	15	15	56
12-5	20	20	57
23-3	15	15	55
27-5	20	20	58
6-7	20	20	60
18-8	20	20	58
AC 9	20	20	59
AC 8b	20	20	60
TA 18	20	20	60
AT 17	20	20	59
GGT 5	15	15	61
GTA 9	15	15	60

4.2.7 Analysis of PCR product

A 10 µl aliquot from the PCR amplified product was analyzed using a 8% polyacrylamide gel in 1X Tris-borate EDTA buffer and electrophoresed at 100v for 5 mins and then at 30v for 16 hrs. An EZ and BG 20 bp and New England Biolab (NEB) or BG 100 bp molecular ruler were run on lane 5 or 6 of twelve-well gel. PCR products were detected by staining the gel with ethidium bromide. Finally the stained gel was destained with distilled water and visualization was done in gel documentation system (Bio Doc, UVP Transilluminator, Upland, USA). The gel image was printed by Sony videographic printer (Sony UP-897 MD, Japan).

4.2.8 VNTR band size determination

The presence or absence of the amplified DNA product visualized on polyacrylamide gel indicated PCR results and further result analysis was done only for PCR positive samples. The distance migrated by the DNA fragment was measured along with migration distances of the 20bp and 100bp molecular weight marker using the measuring tool of Adobe Photoshop (Adobe Photoshop CS for Windows Version 8.0, education Work Version, USA). All data was recorded on an excel spreadsheet (Microsoft Office Excel 2003, USA). A web based server 'Computation of size of DNA and Protein Fragments from their Electrophoretic Mobility' (http://www.imtech.res.in/raghava/dnasize) was used to calculate the exact base pair length of the migrated DNA fragments. Corresponding repeat numbers were identified by correlation with previously described repeat number of tandem repeats present in sequenced TN strain of *M. leprae* (Cole, *et al.*, 2001a).

4.2.9 VNTR allele and cluster analysis

The Microsatellite Tool kit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) was used to calculate allele counts, allele frequency and to format the VNTR data into an Arlequin Frequency and Microsat format. The allele diversity, *H*, for each locus was calculated by Nei's Diversity index (polymorphic information content) [PIC] which is equal to $1-\sum fi^2$ where *f* is the frequency of the *i*th allele (Smittipat, *et al.*, 2005). Clustering was done grouped analysed using three least polymorphic loci namely 27-5, 23-3 and 21-3 as the basis.

CHAPTER - V

RESULTS

5 Recruitment

A total of 52 biopsy samples with BI ≥2 from patients attending Anandaban Hospital were analyzed for the detection of VNTR patterns among *M. leprae* isolates using micro- and minisatellite VNTR PCR. Of 52 processed samples, 48 were from the central region, 3 were from the western region and 1 was from the far western region of Nepal. Regarding ethnicity, Tamang and Chhetri seemed to be predominant ethnic groups within the sample population of patients followed by Magar, Brahmin, Newar, Sunar and others (Table 13).

5.1 Patient demographics

Of the 52 samples, 47 were from males and 5 were from females. The patients were clinically classified according to the Ridley-Jopling system as tuberculoid, borderline tuberculoid (n=1), borderline lepromatous (n=12), lepromatous (n=34), and unclassified (n=5). More than one third of the patients were from the Bagmati Zone (n=36); whereas others attended from Janakpur (n=4), Sagarmatha (n=3), Gandaki (n=1), Narayani (n=5), Seti (n=1), Dhaulagiri (n=1) and Lumbini (n=1) (Table 13).

Table 8. Distribution of samples of patients living within the Bagmati zone of the central region

Districts	No of patients
Kathmandu	7
Bhaktapur	1
Lalitpur	4
Sindhupalchowk	4
Kavrepalanchowk	7
Nuwakot	7
Dhading	6
Total	36

During routine diagnostic and clinical examination, most multibacillary leprosy patients attending Anandaban hospital submit a skin lesion biopsy for analysis by histological examination for Ridley-Jopling classification and for detection of Dapsone or Rifampicin-resistant strains by mouse foot pad culture. Additionally, *M. leprae* collected from biopsy specimens is kept in a frozen repository onsite. *M. leprae* samples were subjected to the freeze and boil method for DNA isolation and then amplified using microsatellite primers for 6 VNTR loci ranging from 181 bp-390 bp and minisatellite primers for 6 VNTR loci ranging from 268 bp -348 bp. Amplicons can be analyzed by both AGE and PAGE. During the start of the study, PCR products were run in 1.5% and 3% agarose but bands were difficult to analyze. Small base pair difference could not be seen. Hence 8% PAGE was used as it has higher resolution power than that of AGE and can separate molecules that differ in size by as little as 2% of their molecular weight (Figure 3).

Forty two of the 52 samples generated positive PCR results for all 12 loci amplified, while 10 samples displayed partial or completely negative results which were confirmed by the repeated PCR for the *M. leprae* DNA. The 42 samples which responded to the complete panel of 12 VNTR loci were then characterized by the number of VNTRs indicated within PCR fragments for further comparison and grouping. Fragment sizes were determined by computer analysis using molecular standards, Adobe Photoshop and a web based programme entitled 'Computation of size of DNA and Protein Fragments from their Electrophoretic Mobility' (http://www.imtech.res.in/raghava/dnasize/). Fragment sizes were then recorded in an excel spreadsheet and correlated with previous reports indicating the corresponding number of VNTR repeats present at each loci with a specific amplicon size. The expected fragment sizes with corresponding VNTR copy numbers are summarized in Appendix III. Subsequent information was further analyzed by the Microsatellite tool kit (http://animalgenomics.ucd.ie/sdepark) to calculate allele frequency, allelic diversity (PIC) and to format the data into an Arlequin frequency and microsat format.

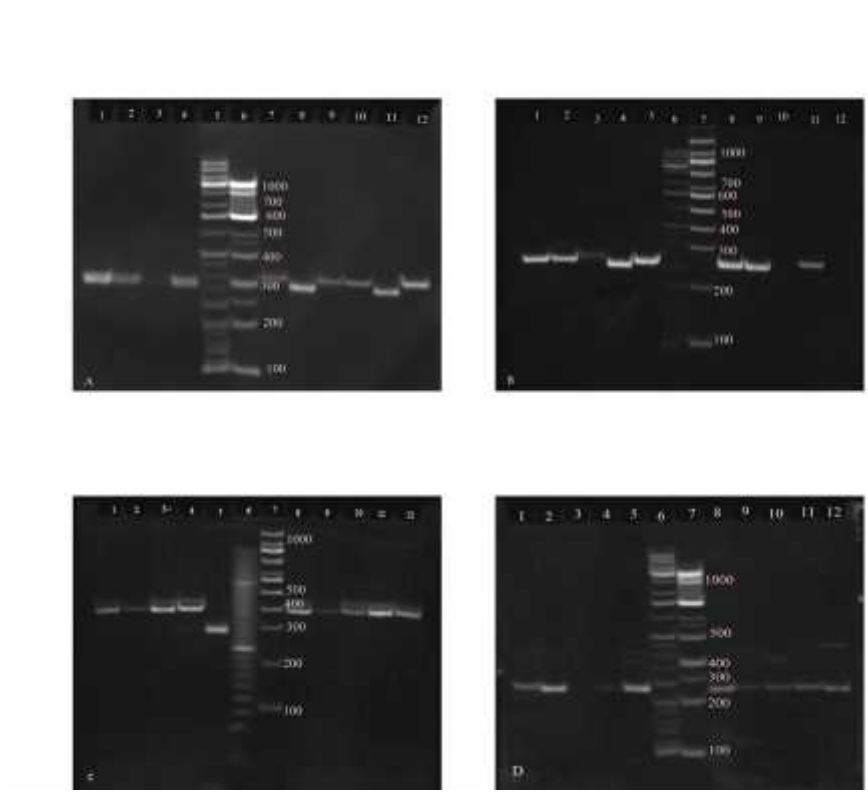

Figure 3. Polyacrylamide gel analysis of VNTR at the minisatellites and microsatellites loci in *M. leprae*

Comment [u15]: I recommend that you combine these four sample gels into one well labeled figure using photoshop. All photos need to be cropped/sized similarly.

In the figure above, A, B, C and D are gel pictures of 21-3, 12-5, AC 8b and GGT 5 VNTRs respectively. Each lane represents a patient sample except for lanes 5 and 6 in A and lanes 6 and 7 in B-D for the 20bp and 100bp molecular standards.

Table 9. Allelic profile of *M. leprae* isolates

Sample ID	27-5	23-3	21-3	18-8	GGT5	12-5	AC9	6-7	GTA9	AC8b	AT17	TA18
1	5	4	5	8	3	3	5	9	15	18	17	17
2	5	3	4	8	4	4	6	9	16	18	12	15
3	5	3	4	8	4	4	11	11	16	13	13	16
4	5	3	4	7	9	3	10	10	13	14	12	15
5	5	3	3	6	2	4	9	15	14	18	13	16
6	5	3	3	6	3	4	8	12	16	20	11	17
7	5	3	3	8	5	3	10	10	17	15	18	17
8	5	3	3	8	5	3	10	11	17	15	15	13
9	5	3	3	8	5	3	4	10	11	16	16	16
10	5	3	3	8	4	4	2	7	11	14	15	15
11	5	3	3	8	4	3	5	10	7	12	18	18
12	5	3	3	8	3	2	10	6	8	13	15	16
13	5	3	3	7	4	2	10	14	7	10	15	17
14	5	3	2	6	9	4	9	10	15	12	13	17
15	5	3	2	9	3	4	8	13	11	16	11	16
16	5	3	2	8	4	2	7	6	8	13	11	18
17	5	3	2	8	4	5	5	7	13	8	7	18
18	5	3	2	6	3	2	10	6	9	12	6	18
19	5	3	1	6	2	7	1	10	13	8	18	10
20	5	3	1	7	2	8	6	6	13	10	19	9
21	5	2	5	8	8	3	9	7	15	14	10	14
22	5	2	4	8	3	3	4	10	14	19	13	13
23	5	2	4	7	5	4	5	10	13	2	18	9
24	5	2	4	7	4	4	5	8	13	21	7	9
25	5	2	3	8	3	2	9	9	12	8	15	17
26	5	2	3	8	3	2	7	9	9	5	17	18
27	5	2	3	8	6	2	8	7	11	10	13	22
28	5	2	3	7	5	2	2	6	9	13	4	15
29	5	2	3	7	2	2	6	10	14	13	15	11
30	5	2	3	7	3	4	6	7	10	10	7	8
31	5	2	3	6	6	3	6	8	10	8	8	20
32	5	1	3	8	6	3	7	8	12	16	8	17
33	4	3	6	8	4	2	11	9	18	12	15	13
34	4	2	5	8	4	3	9	8	16	2	12	18
35	4	2	5	7	5	4	7	10	13	15	17	9
36	4	2	3	8	3	1	7	9	7	10	12	15
37	4	2	3	8	3	2	9	9	7	3	21	19
38	4	2	3	8	4	2	9	8	10	13	17	18
39	4	2	3	8	4	2	6	9	11	20	9	10
40	4	2	2	8	2	3	3	8	13	4	17	14
41	4	3	1	8	4	2	9	5	7	14	21	18
42	6	2	2	8	3	3	11	10	16	13	14	19

Table 10. Allelic properties of VNTR loci in *M. leprae* isolates

VNTR Locus	Copy Number	No of Isolates	Frequency (%)
27-5 (3)	4	9	21.42
	5	32	76.19
	6	1	2.38
23-3 (4)	1	1	1.38
	2	19	45.24
	3	21	50.00
	4	1	2.38
18-8 (4)	6	6	14.29
	7	9	21.43
	8	26	61.90
	9	1	2.38
21-3 (6)	1	3	7.14
	2	7	16.67
	3	21	50.00
	4	6	14.28
	5	4	9.52
	6	1	2.38
GGT5 (7)	2	5	11.90
	3	12	28.57
	4	13	30.95
	5	6	14.29
	6	3	7.14
	8	1	2.38
	9	2	4.76
12-5 (7)	1	1	2.38
	2	14	33.33
	3	13	30.95
	4	11	26.19
	5	1	2.38
	7	1	2.38
	8	1	2.38
AC 9 (11)	1	1	2.38
	2	1	2.38
	3	3	7.14

VNTR Locus	Copy Number	No of Isolates	Frequency (%)
AC9 (11)	4	2	4.76
	5	5	11.90
	6	6	14.29
	7	5	11.90
	8	3	7.14
	9	8	19.05
	10	6	14.29
	11	3	7.14
6-7 (11)	5	1	2.38
	6	5	11.90
	7	5	11.90
	8	6	14.29
	9	8	19.05
	10	11	26.29
	11	2	4.76
	12	1	2.38
	13	1	2.38
	14	1	2.38
	15	1	2.38
GTA 9 (12)	7	5	11.90
	8	2	4.76
	9	3	7.14
	10	3	7.14
	11	5	11.90
	12	2	4.76
	13	8	19.05
	14	3	7.14
	15	3	7.14
	16	5	11.90
	17	2	4.76
	18	1	2.38
AC 8b (15)	2	2	4.76
	3	1	2.38
	4	1	2.38
	5	1	2.38
	8	4	9.52
	10	5	11.90
	12	4	9.52
	13	7	16.67
	14	4	9.52
	15	3	7.14
	16	3	7.14
	18	3	7.14
	19	1	2.38

VNTR Locus	Copy Number	No of Isolates	Frequency (%)
	20	2	4.76
	21	1	2.38
AT 17 (16)	4	1	2.38
	6	1	2.38
	7	3	7.14
	8	2	4.76
	9	1	2.38
	10	1	2.38
	11	3	7.14
	12	4	9.52
	13	5	11.90
	14	1	2.38
	15	7	16.66
	16	1	2.38
	17	5	11.90
	18	4	9.52
	19	1	2.38
	21	2	4.76
TA 18 (13)	8	1	2.38
	9	4	9.52
	10	2	4.76
	11	1	2.38
	13	3	7.14
	14	2	4.76
	15	5	11.90
	16	5	11.90
	17	7	16.66
	18	8	19.05
	19	2	4.76
	20	1	2.38
	22	1	2.38

M. leprae DNA from 42 patient samples were analysed by VNTR PCR and 8% PAGE. The number of repeats identified for each loci were listed for each sample and grouped left to right from greatest to least polymorphic in table 9. *M. leprae* DNA from 42 Nepalese leprosy patients were analysed by VNTR PCR and 8 % gel electrophoresis for minisatellite and microsatellite loci. Allele variants and frequencies determined using 'The Microsatellite Tool Kit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit) was shown in table 10 and the number of allele variants for each locus is given in parentheses after the locus name**.** Locus 27-5 had 3 alleles, 23-3 had 4 alleles and so on in Nepalese population.

Table 11. Allele diversity of minisatellites and microsatellites

Loci studied	Tandem Repeats	Allelic Diversity (h)	Ranking	Conclusion
AC8b	Microsatellite	0.908	1	Very discriminant
AT17	Microsatellite	0.907	2	Very discriminant
GTA9	Microsatellite	0.893	3	Very discriminant
TA18	Microsatellite	0.887	4	Very discriminant
AC9	Microsatellite	0.879	5	Very discriminant
6-7	Minisatellite	0.841	6	Very discriminant
GGT5	Microsatellite	0.780	7	Very discriminant
12-5	Minisatellite	0.722	8	Very discriminant
21-3	Minisatellite	0.687	9	Very discriminant
18-8	Minisatellite	0.550	10	Moderately discriminant
23 -3	Minisatellite	0.543	11	Moderately discriminant
27-5	Minisatellite	0.373	12	Moderately discriminant

Comment [u16]: Is this ranking being interpreted correctly? The microsatellite are not very useful for discriminating strain types. Or maybe this needs to be explained differently.

Of the 12 VNTR loci screened within this study, the minisatellites demonstrated less allelic variability than microsatellites (Table 11). The minisatellite loci 27-5 seemed the least variable within these patient populations as it yielded only three variants, with the majority of the isolates (76.19%) had 5 copy number. Similarly, the minisatellites 23-3 and 18-8 had comparatively less allelic variability with 50% having 3 repeats and 61.9% having 8 repeats respectiviely. Microsatellites, conversely, demonstrated comparative hypervariability with a range of 11-16 allelic variants present within the 42 samples. Microsatellite Tool Kit analysis ranked the VNTR patterns using the mini- and microsatellite results.

Allelic diversity (h) is a good index of the heterogeneity of the sample and discriminatory power provided by the loci under study if the sample is representative of the population

(Sola, *et al.*, 2003). Allelic diversity was calculated as $h = 1-\sum fi^2$ where fi is the frequency of the ith allele at the locus, and n the number of isolates. Based on this index, three VNTRs namely 27-5, 23-3 and 18-8 were designated as 'moderately discriminant' ($0.3 \leq h \leq o.6$). Similarly rest of the 9 loci were designated as 'very discriminant' ($h > 0.6$) is done on the basis of discrimination capacity of genetic marker. It should be discriminant up to the limited level so that it can undoubtedly differentiate the strains. Here in the study microsatellites are observed to be too discriminant as the copy numbers changed frequently yielding large numbers of alleles ranging up to 17 for single loci. So microsatellites can't be considered as suitable genetic marker whereas minisatellite were found to be moderately discriminant and were comparatively stable enough that can be used for strain typing.

Within the limited sample size, the three least variable loci (27-5, 23-3 and 21-3) were selected to attempt molecular sorting of the *M. leprae* samples as they were ranked generally less discriminatory than the microsatellite. Clustering of samples was done on the basis of VNTR pattern exhibited by the isolates which was assigned taking the repeats of three minisatellites 27-5, 23-3 and 21-3 respectively. Seven different clusters (labelled A to G) were identified that included 39 samples and rest of the 3 samples were regarded as unique cluster as they showed unique VNTR patterns (Table 13). Patient geographic and ethnicity data were then compared amongst cluster to identify potential patterns. No apparent patterns using the three least variable minisatellites could be detected according to sex, geographic regions or ethnic groups. All 7 clusters were detected in the Bagmati Zone, the area of greatest sampling in the study.

Table 12. Comparison of 12 VNTRs in Nepalese *M. leprae* isolates with global VNTR data

Loci/	Countries	27-5	23-3	21-3	18-8	GGT5	12-5	AC9	6-7	GTA9	AC8b	AT17	TA18
Strain Types	China[1]	5,6	2	2	6,7,8	4	3	5,7,8,9,10	6,7,8,9,10	9-62	7,8	8, 10-17	11-25
	Nepal	4-6	1-4	1-6	6-9	2-6,8,9	1-7	1-10	1,5-15	7-18	1-21	4-21	8-22
	India[1], Phillipines	5	2,3	1,2,3	7,8	4,5,6	4,5	8,9,10	5,6,7,8,	9,10	7,8		
	Japan[2]	5,3	2,3	1,2,3	7,8	4,5,6	3,4	7,8,10	5-10	8-16, 18	6,7, 8,10	10-16, 17	11-17, 19, 20
	Thailand[2]	5	2	2,3	8	4,5	4	7,8	6	9,11	6,7	11, 13, 15	10, 19, 21
	Korea[2]	5	2	1	7	4	4	7	7	14	8	14	15
	Indonesia[2]	5	2	2	7	5	4	9	6	9	7	13	10
	Malawi[3]		2	1, 2, 3			4, 5		6,7	7-11			
	Brazil[3]									12		20	18
	Mexico[3]									9		14	12
	Ethiopia[3]									8		19	13
	USA[3]									10		18	16
No of alleles	China[1]	2	1	1	3	1	1	5	5	>20	2	>20	>20
	Nepal	3	4	6	4	7	7	10	12	12	>12	>12	>12
	India, Phillipines[1]	1	2	3	2	3	2	3	4	2	2		
	Japan[2]	2	2	3	2	3	2	3	6	10	4	8	9
	Thailand[2]	1	1	2	1	2	1	2	1	2	2	3	3
	Malawi[3]		1	3			2		2	5			
Dominant Allele	China[1]	5	2	2	7	4	3	8	7	12, 9	8	13	18
	Nepal	5	3	2	8	4	2	9	10	13	13	1	18
	India, Phillipines[1]	5	2,3	1,3	7,8	4,5	4,5	9	7,8	8-15	8		
	Japan[2]	5	2	2	7	4	3	7	6	11, 13, 16	8	13	17
	Thailand[2]	5	2	2	7	5	4	7	6	9	7	11,13,15	10, 19, 21
	Malawi[3]		2	2			5		6	9			
M leprae sequenced strain		5	3	3	8	5	5	9	7	9	8	17	18

The allelic variation observed for 12 different loci after VNTR data analysis was compared with VNTR data available for different countries especially India and China as Nepal is leprosy endemic country situated between India and China (Weng, *et al.*, 2007). Comparision of VNTR data of all 12 loci was done with Japanese, Thai, Korean and Indonesian isolates too (Zhang, *et al.*, 2005). For isolates from USA, Brazil, Malawi, Ethiopia, comparision was done only for three microsatellite loci GTA 9, AT 17 and TA 18 (Truman, *et al.*, 2004; Young, *et al.*, 2008)**.**

1= China, India and Phillipines

2= Japan, Korea, Indonesia, Thailand

3= Brazil, Mexico, USA, Ethiopia, Malawi

Table 13. The profile of clustered cases of *M. leprae* isolates

	Cluster →	**A**	**B**	**C**	**D**	**E**	**F**	**G**
1	**VNTR pattern**	5-3-4	5-3-3	5-3-2	5-2-4	5-2-3	4-2-5	4-2-3
	No of isolates	4	9	7	4	8	3	4
	Male: female	2:2	M	6:1	M	7:1	M	M
2	**Bagmati**	3	5	4	4	7	2	3
	Sagarmatha		1	1			1	
	Janakpur		2					1
	Narayani		1	2		1		
	Seti	1						
3	**Aryan: Mongol**	3:1	4:5	3:4	2:2	3:5	2:1	2:2
	Chhetri	3	2	2	1	1	1	1
	Tamang	1	3			3	1	2
	Magar		2	2		1		
	Brahmin		1	1	1	1		
	Mushar		1					
	Newar			2				
	Kami				1			
	Lama				1			
	Sunuwar					1		1
	Parja						1	
	Ghale					1		

Seven different clusters labeled “A” to “G” were identified using MLVA and PAGE analysis comprising 30 Nepalese biopsy-derived *M. leprae* isolates. Each cluster was defined by a specific VNTR repeat pattern identified using the minisatellites 27-5>21-3>23-3, respectively. Cluster VNTR patterns along with the number of isolates and gender ratios are presented within section 1 of the table below. Section 2 lists the geographic distribution of isolates amongst clusters. Section 3 lists the ethnic variation amongst clusters by Aryan: Mongol classification and then separately by caste.

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

The first study in molecular epidemiology of leprosy in Nepal by analyzing Nepalese *M. leprae* isolates using TTC repeats concluded that there were various strains of *M. leprae* in Nepal potentially predominating certain areas and certain ethnic groups; but it was difficult to identify the predominant strains on the basis of a single VNTR (Pandey, 2005). Therefore the present research work was performed using a panel of 12 VNTRs previously reported as useful for analysis in other leprosy endemic areas (Groathouse, *et al.*, 2004). Standard operating procedures for VNTR PCR and PAGE were optimized and assessed for utility of strain identification using facilities available within Nepal.

There are no *M. leprae* strains that exhibit notably different pathogenic or growth characteristics during passage and most strains have been differentiated only according to their geographic origin or donor (Truman, *et al.*, 2004). RFLP analysis as well as sequencing of internal transcribed region of 16S-23S rRNA operon yielded no polymorphism (Truman, *et al.*, 2004). In the past few years, studies focusing on leprosy transmission by molecular genotyping have shed much light on potential use of these methods (Groathouse, *et al.*, 2004; Matsuoka, *et al.*, 2004; Monot, *et al.*, 2008; Truman, *et al.*, 2004; Shin, *et al.*, 2000; Zhang, *et al.*, 2005; Young, 2003; Young, *et al.*, 2004; Young, *et al.*, 2008; Weng, *et al.*, 2006 and 2007).

Molecular typing methods are frequently based on DNA sequence differences between isolates which can arise from single nucleotide changes, deletions and insertions (such as those arising from mobile elements) and repetitive stretches of sequences (tandem repetitions) at different sites of genome (Groathouse, *et al.*, 2004). Short tandemly repeated sequences occur in several to thousands of copies dispersed through the genomes of many, if not all, higher eukaryotes. Hypervariability of STRs are also seen among human individuals hence individual-specific DNA fingerprints can be generated by visualizing these elements with the help of molecular techniques (van Belkum, *et al.*, 1997). Similarly STRs in prokaryotes have been exploited as sources of genetic variation,

particularly for strain typing of species in which inversions, recombination, transpositions are infrequent. Strand slippage during replication has been implicated as the mechanism by which the number of repeats increases or decreases at STRs (Kimura *et al*., 2009). Many reported STRs fall within intergenic regions or within pseudogenes and are therefore unlikely to disrupt any biological pathways; however STRs, particularly the minisatellites, are located within genes. Both microsatellite and minisatellite loci have been selected for this study to evaluate their potential usefulness as molecular typing tools for leprosy epidemiology within the context of Nepal.

VNTR typing is an invaluable tool for genotyping in higher eukaryotes and bacteria as well such as in *B. anthracis* (Keim, *et al.*, 2000), *Yersinia pestis* (Klevytska, *et al.*, 2001), *Francisella tularensis* (Farlow, *et al.*, 2001) *Borrelia burgdorferi* (Farlow, *et al.*, 2002), *Legionella pneumophila* and *M. tuberculosis* (Spurgiesz, *et al.*, 2003). It provides data in a simple and nonambiguous format based on the number of repetitive sequences in so-called polymorphic micro- or minisatellite regions. Although microsatellites also exist in many bacteria, their potential as genetic markers for molecular epidemiology has remained largely unexploited (Mazars, *et al.*, 2001). This fact is at least in part because of the hypervariability of many of these sequences which blurs recognition between epidemiologically linked isolates. The variation generated at VNTR loci provides a high level of subtyping discriminatory power, making VNTRs very useful molecular epidemiological markers but also inhibiting the analytical power for detecting more distant relationships.

Although the *M. leprae* genome contains a number of dysfunctional pseudogenes and has been described as highly degraded (Truman, *et al.*, 2004), it has been shown to be highly conserved. There are a few reports which examined the intraspecies variation in *M. leprae* and nucleotide variation of individual loci such as *RLEP*11, *folP1*12, *rpoB*13 (Truman, *et al.*, 2004). However, none of the above studies could reveal the true level of polymorphism in *M. leprae* isolates but Matsuoka, *et al.* (2000) found polymorphism within *rpoT* gene which was supposed to be the milestone in strain typing of *M. leprae*. Subsequently Shin, *et a.l* (2000) observed that stretch of TTC triplets upstream of a pseudogene exhibited variable number of tandem repeats. In the absence of comparative whole genome sequence data for different isolates, it is essential to have as many

polymorphic loci as possible for the determination of strain variation in this non-cultivable Mycobacteria (Shabanna *et al*., 2003) as discriminatory power of a single VNTR is quite low and the stability of alleles associated with pseudogenes is unclear (Truman, *et al.*, 2004). Taking that into consideration, Groathouse,*et al.* (2004) identified an additional 9 VNTRs namely 6-7, 12-5, 21-3, 27-5 18-8, AT17, TA18, GTA9 and C20 that were subsequently investigated in various populations (Truman, *et al.*, 2004; Zhang, *et al.*, 2005; Weng *et al*., 2006 and 2007); however, results indicated that VNTRs, particularly microsatellites may be too dynamic to use as genetic marker. Meanwhile in some recent studies it has been shown that samples taken from different anatomic sites like skin and nerves on the same patient could yield frequent mismatches indicating that the genotype of the infecting isolate can undergo multiple changes during the evolution of the infection within an individual, or that the patient had been co-infected (Young, *et al.*, 2004 and 2008; Monot, *et al.*, 2008). This potential occurrence of genotypic variation within individual patients points to a need for considerable caution in any application of this type of analysis to tracking of transmission between individuals. Nevertheless, the underlying mechanisms are waiting to be elucidated (Zhang, *et al.*, 2005). Such variability makes short tandem repeat sequences unsuitable as a general tool for population-based strain typing of *M. leprae*, or for distinguishing relapse from reinfection and may be too dynamic for use as epidemiological markers for leprosy. However it remains possible that further repeat loci can be identified as having an intermediate stability suitable for wider transmission tracking (Young, *et al.*, 2008). It is understood that individual infections may not arise from a single strain and hence multiple strain detection within the same patient can complicate epidemiological studies. Therefore further research is necessary to identify which VNTR mini and microsatellites are appropriately discriminatory to serve in a panel for epidemiological investigations within various populations of leprosy patients.

Having porous borders rendering free mobility of people, Nepalese *M. leprae* are expected to share similar VNTR profiles with isolates from neighbouring countries India and China. In this study, similarities were demonstrated and diverse genotypes not previously identified in either neighbouring country were also revealed in Nepal.

Reviewing the global results carried out during different periods of time in India, China, Japan, Philippines, Malawi, USA, Thailand, Indonesia and many other countries, it has been shown that the polymorphism of VNTRs varies with countries. Nevertheless minisatellites like 27-5, 21-3, 23-3 demonstrated conservation of their repeat number intercountries (Zhang, *et al.*, 2005; Truman, *et al.*, 2004; Weng, *et al*., 2006 and 2007). Locus 27-5 locus was found to be non polymorphic with copy number 5 in almost all of the studies carried out earlier but in Nepal, alleles 4 and 6 were also detected. Locus 23-3 in Nepalese isolates exhibited 4 alleles (1, 2, 3 and 4) with 3 being the most common allele. This was in contrast to Chinese, Indian, Japanese, Thai, Korean and Indonesian isolates which exhibited 2 repeats number for the same locus (Weng, *et al*., 2006 and 2007; Zhang, *et al.*, 2005). Copy number 4 of 23-3 locus was rare in other isolates that was obtained in our study. For locus 21-3, copy number 2 was seemed to be common in China, Japan, Indonesia and Thailand whereas Indian and Nepalese isolates shared common repeat numbers 1 and 3 (Zhang, *et al.*, 2005; Weng, *et al*., 2006). Locus 12-5 in Nepalese isolates showed 7 alleles out of which 2 and 3 alleles were common, copy number 4 and 5 were common in Indian samples, 3 in Chinese isolates, 3 and 4 in Japanese isolates, 4 in Thai and Indonesian isolates. For locus 6-7, Copy number 10 was found to be predominant in Nepal although 1, 5 -15 were also observed. Repeat number 6 was dominant in China, India, Japan, Thailand and Indonesia. Similarly 18-8 locus had 4 alleles; 6, 7, 8 and 9 in Nepalese isolates with 8 being the most common. In Indian population, 7 and 8 were common copy numbers whereas 7 was common among Chinese and Japanese population (Weng, *et al*., 2006; Zhang, *et al.*, 2005).

Our study demonstrated that microsatellites exhibited a wide range of polymorphism potentially discriminant within Nepal. Among all six microsatellites, locus GGT5 in Nepalese population was found to be less polymorphic compared to other similar investigations within endemic populations which exhibited up to 7 alleles with allele 4 being most common. Our results contrasted the findings reported from Chinese study with GGT 5 as a non polymorphic locus, although they shared common copy number with Nepalese samples. Indian isolates exhibited repeat number 4 and 5 as dominant ones. Similarly Japanese isolates exhibited 4, 5 and 6 copy number with 4 being common

one. Thai isolates possessed 4 and 5 whereas Korean and Indonesian isolates had 4 and 5 repeats of GGT 5 respectively.

Regarding locus AC 9, it was initially found to be non polymorphic in armadillo derived isolates (Groathouse, *et al.*, 2004) but in other studies, it was found to be widely polymorphic (Weng, *et al.*, 2007; Zhang, *et al.*, 2005). Five alleles of the AC 9 locus were found in Chinese and Thai populations among which 8 was the most common in China and 9 in Indian isolates out of 3 alleles identified (8, 9, 10). Similarly out of 3 alleles (7, 8, 10) of AC 9 in Japanese isolates, 7 was dominant copy number and in Korean isolate as well. Copy number 9 was seen in Indonesian isolates but 10 alleles (1-10) of AC 9 were observed in Nepalese with 9 as most common repeat.

In Chinese populations 53 different alleles of locus GTA 9 was observed among 50 samples studied and 12 was found to be common repeat. Similarly 9 alleles in Indian samples, 10 alleles in Japanese isolates, 2 alleles in Thai isolates were observed with 9 being common copy numbers. Korean isolates had copy number 14 for GTA 9 locus. In Nepalese isolates, 12 different alleles were seen and 13 was the most common repeat number.

Locus AC 8b, AT 17 and TA 18 in Nepalese populations were highly polymorphic showing 15, 16 and 13 numbers of alleles respectively. For locus AT 17, 15 and 17 were common repeat numbers whereas 13 and 18 were common repeats for loci AC 8b and TA 18 respectively. In Chinese, Indian, Korean and Japanese populations, 8 was the predominant copy number of AC 8b locus. Similarly copy number 7 was seen in Thai and Indonesian isolates. Young, *et al* (2004) observed a diverse variety of alleles (8-17 and 19) of AT 17 in Indian populations with 17 being the common allele. Similarly Truman, *et al* (2004) also observed a variety of copy numbers of AT 17 and TA 18 in isolates from Brazil, Mexico, Ethiopia, India Thailand and USA.

Hence the result demonstrated that diverse *M. leprae* genotypes are prevalent in Nepal which is in concordance with prior preliminary findings from MRL (Pandey, 2005). Particular VNTR loci can be polymorphic in one geographical area while comparatively stable in another geographical region indicating potential use of different VNTRs for genotyping within different countries. An important finding was that minisatellites that

were seen to be comparatively conserved in Chinese and Indian population were polymorphic in Nepalese population therefore minisatellites may be more useful for genotyping of Nepalese isolates.

DNA sequencing confirms the presence of VNTR between the isolates and is the best current method for VNTR confirmation. DNA fragment analysis is the state of art of choice for current global studies however; the technology was unavailable within Nepal during our study. So in the present study, an attempt was made to determine if PCR gel elecrophoresis methods were capable of identifying strain patterns within Nepal. In addition, within the current context of Nepal, six minisatellites used in the study may be able to identify major strains.

Amongst the six different minisatellite loci investigated in this study, 27-5 possessed the least allelic diversity value (0.373). Polymorphism observed was categorized arbitrarily into three levels: very discriminant (≥ 0.6), moderately discriminant ($0.3 \leq h \leq 0.6$) and poorly discriminant (<0.3) (Sola, *et al.*, 2003). Loci 27-5 and 23-3 were found to be moderately discriminant; whereas loci 21-3, 12-5 and 6-7 were very discriminant. Remarkably the order of allelic diversity of the minisatellite loci is the same as previously observed in Japanese, Thai, Korean and Indonesian isolates (Zhang, *et al.*, 2005).

Among the six different microsatellites studied, locus GGT5 had the least allelic diversity value (0.78). All 6 microsatellite loci were found to be very discriminant. Loci AT17 and TA18 were much more polymorphic as compared to others. This indicated that particular loci may not be suitable for tracking the strain variability in countries like Nepal although a panel of both minisatellites and microsatellites could be used to type the strains. Small base pair polymorphism should preferably be detected using a DNA sequencer (Zhang, *et al.*, 2005; Weng, *et al.*, 2007).

Although allelic diversity of DNA markers is a common form of strain typing in epidemiology, for STRs to be useful and reliable, polymorphism must remain stable during a few cycles of transmission. On examination of microsatellites panel within Nepalese sample populations, extensive differences were seen indicating that diverse *M.*

leprae genotypes are prevalent in Nepal. However, as expected for such sequences which are highly prone to slipped strand mispairing (Monot, *et al.*, 2005); the level of variability was too great to allow clusters or epidemiological patterns to be detected. Comparing VNTR patterns of *M. leprae* isolates from a particular geographic region with that of another region indicated that different VNTRs could be potentially used for differentiating *M. leprae* from different countries. An important finding was that minisatellites that were seen to be comparatively conserved in Chinese and Indian population were contrastingly polymorphic in Nepalese population. Minisatellites may be useful for genotyping of Nepalese isolates.

Observation over potential bacterial generations within host systems has demonstrated that microsatellites like TTC21 GTA9, AT17 can be stable; whereas, TA18 repeats associated with a putative membrane protein pseudogene showed marked variability among different armadillos inoculated with the same passage preparation (Matsuoka, *et al.*, 2004; Truman, *et al.*, 2004). A marker can be considered to be stable if multiple isolates of an epidemic strain obtained from different patients at different moments are indistinguishable by typing based on that particular marker (van Belkum, *et al.*, 2007). In our study the allelic diversity shown by microsatellites which ranged from 0.780-0.908 is much higher than that of minisatellites which ranged from 0.373 -0.841. The high allelic diversity yielded by microsatellites may not be conducive to smaller population studies; but minisatellite demonstrated allelic diversity within a suitable range for genotyping and potential molecular epidemiological studies (Weng, *et al*., 2006).

As PAGE resolution is limited, these results also highlight that sequencing is more necessary in the case of microsatellites to definitely identify 1or 2 bp differences and copy numbers. Alleles which differ by 4 bases in these 100-350 base amplification products are easily separated in polyacrylamide denaturing gel allowing rapid and precise allele determination. As more extensive base pair differences can be visually analysed by PAGE in developing countries like Nepal with limited resources, minisatellite analysis not requiring sequencing confirmation may prove more functionally reasonable for discerning various strains. It is possible to identify highly polymorphic markers with a relatively low presence of stutter bands. Such markers generally display microvariants and increased mutation frequency. STRs which have high discrimination potential minimal genetic artifacts (such as microvariants) and minimal amplification artifacts

should be selected (Schumm J, 1996), as the presence of such artifacts creates difficulties in results interpretation. Microsatellites like AC9, AT17 and TA18 in this study demonstrated PCR artifacts indicating these may not be suitable markers as compared to minisatellits with comparatively fewer artifacts. Hence in Nepalese populations, these results indicate that initial or broad genotyping of *M. leprae* can be done using minisatellites 21-3, 23-3, 18-8 and 12-5.

Although this study was the first to screen Nepalese *M. leprae* isolates using both mini- and microsatellites, the relative sample size was small in regard to extensive strain identification. However, the sample size in comparision to other global initiatives in other leprosy endemic countries is considerable (Truman, *et al.*, 2004; Zhang, *et al.*, 2005; Weng, *et al.*, 2007). Whereas in other labs a DNA sequencer can now definitively identify genetic differences, within the current context of Nepal, samples had to be analyzed by 12 different PCR with subsequent PAGE analysis. Although this method is labor intensive, this study demonstrates that it can function within Nepal for potential molecular epidemiological studies. As most microsatellites did not yield practically useful data within the context of the study, minisatellites could be used to identify potential clusters within Nepalese *M. leprae* isolates. Microsatellites may yet prove useful in some populations as they are more discriminatory; however, targeted studies of much larger patient populations or populations within well defined areas need to be investigated. In developing countries with limited resources, determining the minimum VNTR panel necessary to discern strains is economically expedient.

6.2 CONCLUSION

Based on this study, minisatellite loci 21-3, 23-3, 27-5, 18-8, 12-5 and 6-7 have potential use in general strain typing as some clustering could be identified demonstrating promise that further studies may allow for molecular discernment of strain trends between ethnically or geographically diverse isolates. Specifically, microsatellites AT 17, TA 18, AC 8b, GGT 5 allelic trends were shown to be more or less variable within Nepalese patient populations than in global arena (Weng *et al*., 2006 and 2007). No geographical or ethnic relationships amongst molecular clusters in Nepalese populations were evident; however, larger population studies would be necessary for proper assessment.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

Comment [TLM17]: We'll work on this some more later. After the results and discussion parts are reshaped.

7. 1 SUMMARY

Fifty two Nepalese leprosy patient skin biopsy samples were processed for *M. leprae* DNA extraction by the freeze and boil method and then analyzed by MLVA PCR assessing 6 minisatellite and 6 microsatellite VNTR loci using sequence specific primer sets. Of 52 samples, only 42 samples gave PCR positive results for all 12 primers and were further processed for result analysis and interpretation. All 12 loci demonstrated a wide range of allelic diversity ranging from 0.373 to 0.908. Minisatellite 27-5 was found to be least variable with allelic diversity 0.337; whereas microsatellite AC8b was found to be most variable with allelic diversity 0.908. Although allelic diversity of DNA markers is the basis of strain typing, high diversity within preliminary smaller population studies may not be as useful for epidemiological typing. Although minisatellites were here in shown capable of identifying major clusters, the methods available within Nepal for minisatellite VNTR detection may not be sensitive to small base pair differences between isolates. Microsatellite allele differences could not be reliably resolved using PAGE analyses and may best be analyzed when a DNA sequencer is available.

Allele differences for loci 27-5, 21-3 and 23-3 successfully differentiated the isolates into 7 distinct clusters. No correlation between the molecular strains was detected according to region, ethnic group or sex. Comparison of Nepalese VNTR profiles with global isolates profiles from geographically distinct areas revealed that Nepalese *M. leprae* populations are relatively diverse and potentially harbor distinctive strains.

7.2 RECOMMENDATIONS

The present study was carried out with an aim to address key questions regarding VNTR typing for strain differentiation of *M. leprae* isolates from Nepal. The study characterized the allelic diversity of loci within the sample population, their discriminatory power and the potential suitability of these loci to be used as genetic markers to type Nepalese isolates. Some queries in the study could be used as guidelines for future research projects. Considering the practical limitations of limited sample size for MLVA for field

based molecular epidemiology, recommendations made from this research work are as follows:

1. Minisatellite loci 21-3, 23-3, 27-5, 18-8, 12-5 and 6-7 should be useful for strain differentiation in Nepalese populations which need further studies to confirm their relevance to global molecular trends.

2. The optimized VNTR PCR protocol can be used to carry out larger scale MLVA study within defined endemic geographical areas or within multicase families to potentially help understand molecular epidemiology and short range transmission patterns of *M. leprae* in Nepal.

3. Some minisatellites seemed to be polymorphic in Nepal that were non polymorphic in Japan, Thailand, China and India. These should be confirmed by more advanced analyses using a DNA sequencer. Further studies of larger populations should be performed in Nepal to better characterize the VNTR profiles within the global context of leprosy.

4. Minisatellite analysis using PCR and PAGE analysis is capable of identifying potential strain clusters within Nepalese *M. leprae* isolates. It has therefore been demonstrated as feasible within areas without access to a DNA sequencer to differentiate potential strains.

5. Microsatellite analysis may not be feasible in small population studies due to high diversity or in areas with limited access to a DNA sequencer. The small base pair differences (1-3bp) are difficult to definitively resolve by gel electrophoresis alone and require DNA fragment analysis for validation.

Comment [TLM18]: When using a highly discriminatory test, the sample size was far too small to draw too many conclusions on th eusefulness of microsatellites in Nepal. For practical reasons (sequencing required), microsatellites may be limited in their application in the field; therefore, if a panel using minisatellites VNTRs discernable by PCR alone is developed, that may have more immediate application with less expense in Nepal.

CHAPTER-VIII

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APPENDIX I

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1. Materials

Acrylamide- **SIGMA, Germany**

Acetone-

Ammonium per sulphate

Boric acid

Bromophenol blue-**SIGMA, USA**

Double processed tissue culture water-**SIGMA, USA**

Ethidium bromide

Ethanol

Ethylene Diamine Tetra acetic Acid (EDTA)

Hypochlorite (20%)

Magnesium Chloride (MgCl2)-**Bangalore Genei**

Molecular weight Markers (20bp and 100bp)-**Bangalore Genei, New England Biolab**)

Nucleotides dNTPs (dATP, dCTP, dGTP, dUTP)-**Bangalore genei**

PCR buffer (10x) **-Bangalore Genei**

Phosphate buffered saline (PBS)

Sucrose

Taq polymerase-**Bangalore genei**

Tris Borate Buffer (TBE)

Tetra ethyl methylene diamine (TEMED)

Trizma base

2. Glasswares and Plasticwares

Beaker andmeasuring cylinder

Conical and volumetric flasks

Glass plates with fixed 1mm spacer

Notched glass plate with straight edge

Cuvette

Disposable tube

Clamps and rubber

Equipments	Company
Dry Heat Block	DB2A Techne UK
Spectropotometer	8625 UNICAM, UK
Biosafety Cabinet Class II	BH12Labcaire System Ltd., UK
T3 Thermocycler	Whatman Biometra Germany
Vertical Gel Apparatus	Whatman Biometra Germany
Power supply	PS250 Hybaid, UK
Gel Documentation System	UVP-upland, USA.
Videographic Printer	Sony UP-897MD, Japan
Vaccum Machine	Ashcroft, USA.
Analytic Microbalance	AE240 Mettler, Switzerland
PH meter	PH90 WTW.
Vortek shaker	VM-300,Gemmy, Taiwan
Mini centrifuge	Techne, Cambridge. UK

3. Pippetes and tubes:

Plastic tubes 0.5ml, 1.5ml (Eppendorf tube, Treff)-

Yellow cap tube 15ml

Falcon tubes 50ml -Greiner

PCR tubes 0.2ml -Molecular bioproducts, USA

Micropippete 10µl, 20µl, 50µl,200µl, 1000µl-Biohit

Pipette tips 5 and10 ml-

4. Miscellaneous

Forceps

Gloves (sterile and nonsterile, Surgicare India)

Markers

Paraffin paper

Soaps

Tube holder

Tissue paper

Digital Timer

APPENDIX II

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Minisatellites and Microsatellites in *M leprae* TN genome [a]The co-ordinates, repeat units and the gene numbers are as for the *M. leprae* TN genome sequence (Jones *et al*., 2001). * stands for pseudogenes.Each locus was given an ID for easy reference (length of repeat number of repeats followed by a letter if needed).

Locus ID	**Repeat unit (bp)**	**Coordinates(Chromosome Region)**	**Intergenic Region**	**Intragenic Region(Gene orientation)**
27-5	27	687026-687160		ML0568/+
23-3	23	2945487-2945555	ML2469-2470*	
21-3	21	73077-73139		ML0058/-*
18-8	18	1587619-1587763		ML1134
12-5	12	1381661-1381723		ML1182
6-7	6	1816857-1816892		ML1505
AC 8b	2	2211035-2211050	ML1824-ML1825	
AC9	2	1452573-14525590	ML1227-ML1228	
AT 17	2	2597735-2597768		ML2183/-*
TA 18	2	984591-984626		ML0830/-*
GTA 9	3	2583814-2583840	ML2172-ML2173	
GGT5	3	2567251-2567265	ML2159-ML2160*	

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APPENDIX III

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Expected base pairs and its corresponding repeat of each locus were calculated on the basis of tandem repeats on sequenced strain of *M. leprae*.

Loci	Expected base pairs	Expected Repeats
	Minisatellite	
	300	4
	327	5
27-5	354	6
	291	2
	312	3
21-3	333	4
23-3	303	2
	326	3
	312	6
	330	7
18-8	348	8
	253	2
	265	3
12-5	277	4
	289	5
	262	6
	268	7
6-7	274	8
	280	9
	286	10
	Microsatellite	
	233	2
	236	3
GGT 5	239	4
	242	5
	234	7
	238	9
AC 9	240	10
	301	7
	304	8
GTA 9	307	9
	310	10
	313	11
	316	12

Loci	Expected base pairs	Expected Repeats
	319	13
	322	14
	325	15
	328	16
	331	17
	334	18
AC 8b	390	8
	394	10
	400	13
	402	14
	404	15
	406	16
	410	18
TA 18	224	15
	226	16
	228	17
	230	18
AT 17	171	12
	173	13
	177	15
	181	17
	183	18