2 REVIEW OF LITERATURE

2.1 History

Leprosy is an ancient disease known to mankind for thousands of years. The clinical evidence of the disease dates back to Vedic times in India as well as Biblical times in the Middle East. An Egyptian skeleton dating back to 2nd Century BC shows unambiguous evidence of leprosy (Dzierzykry-Rogalski 1980, Lechat 1999). The earliest written records of leprosy from Greece date back to the 3rd century BC while inscriptions from China date back to 600BC (Anderson JH 1969, Jopling and MacDougall 1988, Kaplan DL 1993). In India, the earliest descriptions of leprosy are found in manuscripts dated 600BC, when leprosy was described as Kushtharoga (in Sanskrit: eating away). Atharvaveda (2000BC) (Rogers and Muir 1940) and laws of Manu (1500BC) describe various changes suggestive of leprosy. Sushrut Samhita provides a detailed account of clinical features of leprosy (Dharmendra 1969).

2.2 Causative Organism

*Mycobacterium leprae*, the causative organism of leprosy was identified by Gerard Armaeur Hansen, a Norweigian physician in 1873. It is an obligate intracellular rod-shaped bacterium. The cell wall has high
molecular weight mycolic acids which renders the bacilli resist to
decolorization when using Zeil Neilsen Carbol Fuschin method and hence
its acid fast nature. It is on basis of this acid-fast nature that the
Hansen’s bacillus was included in the genus *Mycobacterium*, together
with other closely related species like *Mycobacterium tuberculosis* and
classifies the organism as: Class II – Schizomycetes; Order VI –
Actinomycetales; Family II – Mycobacteriaceae; Genus –
*Mycobacterium*; Species – *M.leprae*. The capsule is composed of
Phenolic glycol lipid-1 which is specific to *M. leprae* and other
molecules like PDIM, Phosphatidyl Inositol mannosides (PIM) and
phospholipids. The cell membrane contains phospholipids, proteins,
and Major Membrane Protein –I (MMP-I) and Major Membrane Protein–
II (MMP-II) (Cho et al 1983)

Despite various attempts by scientists over the last 125 years, *M.
leprae* has not been cultivated *in vitro* in an artificial media. However it
can be maintained in axenic cultures in a stable metabolic state for a
few days. Propagation of *M.leprae* has been restricted to animal
models like normal athymic mice and armadillo (Krahenbuhl 2000,
Truman et al 2001). This obligate pathogen has been shown to
maintain its metabolic activity in axenic medium cultured murine and
human macrophages and primary rat Schwann cells for several weeks
at 33°C but loses its activity at 37°C.
2.3 Magnitude of leprosy problem:

Despite the advances in clinical and basic biological sciences in the last century, this ancient disease still remains a major concern in countries like India, South America, Central Africa, and Southeast Asia. Prevalence for leprosy is defined as the number of leprosy cases per 10,000 people in the population. Widespread use of Multiple Drug Therapy (MDT), provided by the World Health Organization (WHO) has drastically reduced the global prevalence from 12 million cases in 1985 (WHO) to a registered prevalence of 211,903 at the beginning of 2009 (WHO Report 2009). In India, leprosy was considered to be at its peak with a prevalence rate (PR) of 57/10,000 population in 1981. By March 2009, it had fallen to 0.72/10,000 (NLEP). Though there is a reduction in the prevalence, a high number of new cases are being detected indicative of ongoing transmission.
Figure 2.1: Global leprosy PR at the beginning of January 2009

(http://www.who.int/lep/situation/LEPPRATEDJAN2009.pdf)

Graph 2.1: Trend of leprosy Prevalence and ANCDR (Annual New Case Detection Rate) in India

Graph showing decrease in PR and ANCDR from 1991 to 2010.  
(Adapted from NLEP).
2.4 Immunological Overview:

2.4.1 Clinical features and immunological effects

During infection leprosy affects the skin, nerves, and eyes of patients. Many patients present with anaesthetic skin lesions (hyper or hypo pigmented), enlarged nerves, weakness and numbness caused by peripheral nerve damage, nerve pain, eye pain, or sometimes with ulcers in an anaesthetic foot or hand (Walker et al 2006). Patients may also present initially with a reaction, which is a common complication. This obligate intracellular bacterium lives within the phago-lysosome of macrophages, peripheral Schwann cells, and epidermal keratinocytes.

2.4.2 Reactions in leprosy:

Reactions in leprosy are as a result of spontaneous fluctuations in the clinical state due to the dynamic nature of the immune response to *M. leprae* (Britton et al 2004). Around 20-30% of the patients develop acute reactions during or after the course of the disease. There are 2 types of reactions: Type I reactions and Type II reactions. Type I reactions (T1R), also called as Reversal Reactions (RR), occur in borderline spectrum of the disease and are characterized by delayed type hypersensitivity (DTH) with increased CMI towards *M.leprae* (Godal et al 1973). Type 2 reactions, erythema Nodosum Leprosum(ENL), is an immune complex mediated vasculitis common
in LL and BL leprosy. It usually manifests as red nodules or papules in the skin and may also involve nerves, lymph nodes, joints eyes and testes.

**Figure 2.2** Schematic diagram of Immunopathological presentation of leprosy

As shown in the above figure Ridley Jopling has classified leprosy in a 5 point spectrum depending on clinical and histological features

**2.5 Classification Methods:**

**2.5.1 Ridley Jopling Classification:**

Interestingly, the varying clinical manifestation of leprosy is governed by the host immune response to the bacilli. On exposure to *M. leprae* and the host immunity, a single patch, which is typically called indeterminate, may self heal or disease may establish itself in the host. If *M. leprae* establishes an infection, the patient then subsequently falls into one of the 5 types. In this model (Figure 2.2)
stable lepromatous (LL) and tuberculoid (TT) forms are found at the polar ends of the clinical spectrum, while clinically unstable forms are found between the poles. These unstable forms include mid-borderline (BB), Borderline tuberculoid (BT) and Borderline lepromatous (BL) (Ridley 1964; Ridley & Jopling 1966).

Typically indeterminate leprosy appears as one or more slightly hypopigmented lesions which may heal spontaneously depending on the host immunity or may progress towards one of the polar forms of leprosy (TT or LL) or exist in one the borderline states (BT, BB, BL) (Dharmendra 1969). Tuberculoid leprosy patients have strong Cell Mediated immunity (CMI) but low antibody levels. This is reflected in granuloma formation in nerves and paucity of bacilli in the lesions. Patient presents with slight to markedly thickened nerves and well defined anaesthetic patches which are very well demarcated from surrounding normal skin. The cutaneous surface is often dry and there may be loss of hair with explicit loss of cutaneous sensibility and thickened nerves in the patches (Dharmendra 1969).

Lepromatous leprosy patients have high humoral immunity and weak cell mediated immune response to M.leprae antigens. Mycobacteria proliferate within macrophages the infection is disseminated and results in multiple lesions that are symmetrically distributed throughout the body. The margins of each lesion are not well defined thus; there is no clear demarcation from normal skin. These nodules are highly loaded with M. leprae and the routine slit skin smears are
positive for Acid-Fast Bacilli (Dharmendra 1969).

### 2.5.2 WHO Classification:

The 5-point Ridley Jopling classification was meant solely for research. In 1982 WHO recommended an operational classification for treatment purpose. This simplified classification system allowed leprosy diagnoses in the field and facilities where histological evaluation is not accessible. The WHO Classification system is based on the number of *M. leprae* harboured by an individual and the number of skin lesions. All the patients with Bacillary Index (BI) $\geq 2$ and greater than 5 lesions are termed ‘Multibacillary’ (MB) and those with BI $< 2$ and less than 5 lesions are termed ‘Paucibacillary’ (PB) (WHO 1982). The classification was further revised in 1988 wherein all smear positive cases were grouped as MB whereas all Indeterminate, borderline and Tuberculoid cases (except those with BI positive) were termed PB. In 1998 as leprosy diagnosis became integrated into public health sector and with the unavailability of slit skin smear facilities at field sites, the 7th WHO expert committee recommended a much simpler classification. To reiterate, this new classification groups patients as follows:

1. **Paucibacillary (PB)** 1-5 lesions
2. **Multibacillary (MB)** $> 5$ lesions

### 2.6 Diagnosis and Treatment

Diagnosis of leprosy for purpose of treatment is based on several
important clinical findings. These include physical examination, bacterial index through slit skin smear and histological evaluation (as and when required).

2.6.1 Physical examination:
The cardinal signs for the physical examination and clinical diagnosis of leprosy are anesthetic patches, and thickening of nerves. Diagnostic criteria for leprosy are based on patients having one or more of the three cardinal signs:

- hypo pigmented or hyper pigmented patches
- loss of sensation in patches
- thickened peripheral nerve(s)

2.6.2 Laboratory Diagnosis: Slit Skin Smear
In addition to the physical examination, the bacterial quantity indicates disease severity. Slit skin smears are used to identify the patients with a high bacterial load and are thus most infectious. In this technique, fluid is collected from various sites on the body (ear, eyebrow, etc) and evaluated for bacterial quantity (index). *Mycobacteria* bacilli can be viewed through Zeil-Neilson staining. Bacteriological index (BI) is an objective way of monitoring the benefit of treatment and is graded by Ridley-Jopling scale of zero to 6+ (Ridley et al 1964). The mean BI of an individual is calculated by totaling the score of each site and dividing it by the number of sites from which it is collected. A minimum of 4 sites are examined for each patient. In paucibacillary leprosy the bacterial index is usually negative while it
ranges from negative to 4+ in multibacillary leprosy patients.

2.6.3 Treatment: Treatment for leprosy often depends solely upon clinical diagnosis and the diagnostic factors discussed above. The first line drugs used for the treatment of leprosy are rifampicin, clofazamine and dapsone. Based on the type of leprosy, the drugs, doses, and duration of treatment vary (Table 1).

Table 2.1: Standard WHO regime for an adult leprosy patient

<table>
<thead>
<tr>
<th>Type</th>
<th>Skin lesion/BI</th>
<th>MDT</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>&gt;6 skin lesions</td>
<td>Rifampicin</td>
<td>600mg/month</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BI positive</td>
<td>Dapsone</td>
<td>100mg/daily</td>
<td>12 months</td>
</tr>
<tr>
<td></td>
<td>Clofazamine</td>
<td>&amp; 50mg/daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>2-5 skin lesion</td>
<td>Rifampicin</td>
<td>600mg/month</td>
<td>6 months</td>
</tr>
<tr>
<td></td>
<td>BI negative</td>
<td>Dapsone</td>
<td>100mg/daily</td>
<td></td>
</tr>
</tbody>
</table>

MB- Multibacillary; PB: Paucibacillary

To prevent permanent nerve damage and deformity adequate treatment is administered for patients with RR and ENL. Severe ENL, RR, and neuritis are treated with prednisolone for 12 weeks course starting with 40-60mg/ daily and gradually reducing the dose weekly. Mild ENL is treated with non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin. For severe ENL, Clofazamine dosage may
be increased up to 100mg/daily. For more severe or recurrent ENL, thalidomide may be given for a period of 12 weeks with tapering dose.

### 2.7 Genome of *M. leprae*:

*M. leprae* genome sequence got initiated in 1991 and the whole genome sequence was deciphered in 2001 for an isolate from Tamil Nadu. The circular genome is 3.27Mb (3.268, 203 bp) in size and has an average G+C content of 57.8% (Cole et al 2001). These values are much lower than those reported for the *M. tuberculosis* genome, which is 4.4Mb in size with a 65.6% G+C content (Cole et al. 1998, Tekaia et al 1998). Table gives the comparison between *M. leprae* and *M. tuberculosis*.

There are 1614 coding genes open reading a frame (ORF’S) as compared to 3993 ORF’s predicted in *M. tuberculosis*. One of the most remarkable features of the *M. leprae* genome is that it possesses 1,133 pseudogenes (genes lost through mutation and active functionally in *M.tuberculosis*), compared to 6 pseudogenes in *M. tuberculosis*. The remaining 23.5% of the genome does not appear to be coding and may correspond to regulatory sequences or even gene remnants mutated beyond recognition. Interestingly, though the average G+C content is 57.8%, the G+C content of *M. leprae* functional genes (60.1%) is higher than that of pseudogenes and the remainder of the genome. There are fewer insertion sequences but more repetitive DNA
sequences (RLEP, REPLEP & LEPREP) in the *M. leprae* genome (Cole et al 2001).

*M. leprae* contains the highest number of pseudogenes among genomes published to date (Harrison et al 2002, Liu et al, 2004). Several attempts have been made to explain the basis of this exception. The possible causes for the increase in pseudogenes in *M. leprae* has been attributed to the loss of the sigma factors. A loss of dnaQ-mediated proofreading activities of DNA polymerase III might also contribute to a high mutation rate (Liu et al., 2004). However, because pseudogenes are supposedly eliminated from the genome due to deletion events, their accumulation in *M. leprae* requires further explanation. One group has postulated that because of its intracellular lifestyle, *M. leprae* has a low level of exposure to potentially detrimental DNAs, such as horizontally transferred DNA (e.g. bacteriophages and transposons), resulting in the suppression of chromosomal deletions and the accumulation of pseudogenes (Lawrence et al 2001). Despite these *in silico* studies, there was no evidence showing the actual expression or the possible role of *M.leprae* pseudogenes until recently with the first whole genome- wide expression profile of *M.leprae* genes demonstrated a high level of expression of pseudogenes and non-coding regions (Suzuki et al 2006, Akama et al 2009). To summarize, it seems that this obligate intracellular parasite has just the minimal set of genes required for survival in a macrophage, Schwann cell or keratinocyte.
Table 2.2: Comparative genomics of Mycobacterium leprae and Mycobacterium tuberculosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>M. leprae (strain TN)(^{a})</th>
<th>M. tuberculosis (Strain H37Rv)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMBL/GenBank/Accession no</td>
<td>AL450380</td>
<td>AL123456</td>
</tr>
<tr>
<td>Genome size (bp)</td>
<td>3,268,203</td>
<td>4,410,532</td>
</tr>
<tr>
<td>No. of protein genes</td>
<td>1,614</td>
<td>3,993</td>
</tr>
<tr>
<td>No. of unknown genes</td>
<td>142</td>
<td>606</td>
</tr>
<tr>
<td>No. of Pseudogenes</td>
<td>1,133</td>
<td>6</td>
</tr>
<tr>
<td>No. of tRNA genes</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>No. of rRNA genes</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. of Stable RNA genes</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gene density (bases/gene)</td>
<td>2,024</td>
<td>1,106</td>
</tr>
<tr>
<td>Average gene length (bases)</td>
<td>1,007</td>
<td>1,008</td>
</tr>
<tr>
<td>% Protein coding</td>
<td>49.5</td>
<td>91.2</td>
</tr>
<tr>
<td>% G+C</td>
<td>57.8</td>
<td>65.6</td>
</tr>
<tr>
<td>SNP frequency</td>
<td>1 in 24000 bp</td>
<td>1 in 3000 bp</td>
</tr>
</tbody>
</table>

(a) Data obtained from the Current Data Release (17 Oct. 2003) for the M. tuberculosis genome (http://genolist.pasteur.fr/TubercuList)
(b) Data obtained from the Current Data Release (20 July 2005) for the M. leprae genome. (http://genolist.pasteur.fr/Lepromat/)
2.8 Genotyping of *Mycobacterium leprae*:

2.8.1 Molecular tools:

Standard clinical and immunological tools have limited value for diagnosis of leprosy at early stages and for monitoring treatment while the availability of *M. leprae* genome sequence led to the development of molecular techniques that could confirm cases missed by histological evaluation. During the last 3 decades rapid molecular assays have been applied for the amplification of *M. leprae* from clinical specimens (Katoh 1991, Richter 1994). Several genes encoding *M. leprae* proteins such as 18KD (Clark-Curtiss et al 1989, Williams et al 1990), 35KD (Hartskeel et al 1989), 65KD (Plikaytis 1990), leprosy serum reactive protein (LSR) (Mishra et al 1995), Ag85 complex (Hackel et al 1990), 16SrRNA (Cox et al 1991, Pattyn et al 1992), and repetitive sequences (Clark-Curtiss et al 1989, Jamil et al 1994) were amplified by polymerase chain reactions. RT-PCR targeting 16SrRNA (constitutively expressed gene that codes for the 16S portion of the bacterial ribosome) (Pattyn et al 1992, Kurabachew et al 1998, Jadhav et al 2006) has been reported to be useful for monitoring viable bacilli in clinical samples. Nucleic Acid Sequence Based Amplification (NASBA) has been used for detection of viable *M. leprae*. Real time PCR and RT_PCR have been used for quantification of DNA and RNA, respectively from slit skin smear and biopsy (Kurbachew et al 1998, Kramme et al 2004, Martinez et al 2006). Various targets like *M. leprae* hsp18 mRNA – to monitor chemotherapy (Lini et al 2009) and RLEP
(Repetitive sequence) are found to be very sensitive (Truman et al 2008).

2.8.2 Transmission of *M. leprae:*

There exist two schools of thought favoring the respiratory or dermal sites of entry and exit of *M. leprae* (Davey et al 1974, Rees et al 1976). As regards to the nasal route, there is a preponderance of reports as early as the 1950s indicating that the nasal mucus is the primary site of entry and discharge of *M. leprae* (Rees et al 1976). Large numbers of bacilli in nose-blow of leprosy patients which are reduced during and after treatment have been reported (Davey et al 1974). McDougal *et al.* described in detail the distribution of *M. leprae* in nasal tissues and compared the histopathology of biopsies obtained from LL and TT patients (McDougall *et al.* 1975). Changes in the histology of the nasal mucosa have been described even in pure neuritic leprosy patients (PNL) that have no skin lesions (Suneetha *et al.* 1998).

*M. leprae* adheres to nasal epithelial cells (Byrd *et al.* 1993) as demonstrated in *vitro,* which could result in the colonization of nasal mucosa. This state of *M. leprae* carriage, as demonstrated by the detection of *M. leprae* DNA in nasal swabs by PCR based DNA amplification or anti PGL-I antibodies in nasal swabs is common and has been used as an indicator of recent transmission within some populations (Beyene *et al.* 2003).
2.8.3 Genetic Variations in *M. leprae*:

Molecular tools can help in devising techniques for understanding the epidemiology of leprosy and identifying sources as well as causes of persisting foci of disease. Molecular methods to elicit strain differences within the leprosy bacillus would be especially important for this purpose. Molecular typing will make it feasible not only to study the global and geographical distribution of distinct clones of *M. leprae*, but also to explore correlation between the *M. leprae* and the type of disease manifested and provide insight into historical and phylogenetic evolution of the bacillus. (Monot et al 2005). Ultimately these genetic markers may hold the key to establish species and strain specific markers for identifying the sources of *M. leprae* and tracing transmission patterns.

Interestingly the complete genome sequence of *M. leprae* also revealed psuedogenes, repetitive sequences and tandem repeats. Various genotyping methods have been employed to study the strain variation in *M. leprae*. Restriction fragment length polymorphism (RFLP) analysis of *M. leprae* isolates using a combination of restriction enzymes and probes and sequencing of the internal transcribed spacer region of the operon of 16S-23S rRNA revealed no polymorphic DNA sequences (Clark-Curtiss et al 1989, Williams et al 1990, de Wit et al 1994). A polymorphic structure in the *polA* gene was reported to be associated with genotypic variation in *M. leprae* (Fishi et al 1995).
2.8.4 Variable Number of Tandem Repeats for genotyping

Whole genome sequencing of *M. leprae* revealed tandem repeats scattered through the genome either in intragenic, intergenic regions or within pseudogenes that could be potential markers for genotyping *M. leprae*. Short tandem repeats (STR) are patterns of two or more nucleotides, which are repeated in the genome and are placed adjacent to each other. These STR vary in copy number creating polymorphisms of variable lengths. Variable numbers of tandem repeats (VNTR) are tandem repeats sequences that are repeated multiple times which vary in copy number in different strains creating variable length polymorphism. Primers flanking the variable region can be designed for amplification and sequencing. (STR) based polymorphisms have been used as a powerful typing tool for differentiating several organisms (Klevytska et al 2001, Keim et al 2000, Supply et al 2003). Various strain typing methods like VNTR have been used for genotyping studies of *M. tuberculosis* (Filliol et al 2000). The simultaneous analysis of such STR loci can be used to gain insight into the diversity of *M. leprae* isolates, its evolution, and pathogenicity. First Tandem repeat studied was *rpoT* in 2000 followed by TTC, a trinucleotide repeat. With these preliminary findings, the genome of *M. leprae* was screened in-silico and subsequently identified 11 STR loci were identified *M. leprae* DNA from four human isolates was passaged through armadillos. Of the 11 STR loci that were tested, 9 were found to be polymorphic – (Groathouse et al
2004). With this limited sample set, a third study used five polymorphic minisatellites (with repeat units of 6-50 base pairs) and four microsatellites (1-5 bp repeat units). Use of one to three microsatellite markers uncovered a large number of disparate genotypes, which could not be grouped into clusters for the identification of transmission chains (Matsuoka et al 2004, Young et al 2004). Allelic diversity in DNA markers is the basis of strain typing in epidemiology. Yet, to be useful for epidemiological studies reliable polymorphisms must remain stable during transmission. It was demonstrated that microsatellites (AT)17, (TA)18, (GTA)9, and (TTC)21 were highly stable after multiple passages in armadillo and/or nude mice, even though allele shifts by one or more repeat units were not infrequent (Truman et al 2004). Also 16 polymorphic loci when assessed for sensitivity, specificity and reproducibility in standard assays using an *M. leprae* reference strain were found to be highly stable (Gillis et al 2009).

VNTR's are classified based on the number of repeat unit as follows:

- Minisatellites are nucleotide repeats of 4-5 bases
- Microsatellites nucleotide repeats of 6-50 bases
Some of the loci studied for strain typing are:

- **pol (A) gene** encoding DNA polymerase, is flanked by two inverted copies of a new class of the *M. leprae* specific repetitive sequence, RLEP& this structure resembles a transposable element. A second of this element was found at another locus in the genome but the two copies were not present in equal amounts & could not be found in another isolate of *M. leprae* (Fsihi & Cole 1995). No variation in polA gene was observed in North Indian population as all the strains exhibited 2 copies of this gene (Lavania et al 2011).

- **rpoT**: This is a hexanucleotide repeat (GACATC) in the intragenic region of rpoT gene. This was the first tandem repeat which exhibited polymorphism with predominantly two alleles in different populations (Matsuoka et al 2000). Some strains from Japan carried three copies while others carried four copies (from Brazil, Haiti and Okinawa) (Matsuoka et al 2000 & 2005). However isolates from Northern India, predominantly exhibited 3 repeats in rpoT gene (Lavania et al 2007).

- **(TTC) 21**: The second highly polymorphic loci was a tri-nucleotide repeat (TTC) present upstream of a pseudogene in the non-coding region of genome of *M. leprae*. TTC tri-nucleotide repeats were very specific to *M. leprae* as these were not found in *M. tuberculosis, M. avium*, or *M. marinum* or human tissue (Shin et al 2000). In this study, the copy number of the TTC
triplet varied from 10 to 37 in different clinical isolates and the most common had 24 and 25 repeats. Different clinical isolates exhibited high variability of TTC repeats. A study from Mali reported 10-17 copy number of TTC with 14 being the most common repeat (Monot et al 2008). Earlier study from India confirmed the diversity of TTC repeat (Young et al 2004) with copy number ranging from 11 to 20. Yet another study from North India on 300 patients reported variation in the copy numbers (6-20) with >80% of them exhibiting 10-20 copies (Lavania et al 2011). Sampling from various sites was done and it was reported that the TTC repeats were highly variable in the population.

- **AT (17)**: This is located in intragenic region within a conserved hypothetical pseudogene and TTC loci. The TN strain has 17 repeats of this dinucleotide. Alleles for these loci showed variation from 10-15 repeats in North and South American isolates (Truman et al 2004). Study from Mali showed 9 alleles for AT(17) loci but the most common was 13 (Monot et al 2008)

- **TA (18)**: TA18 is associated with a putative membrane protein pseudogene and is located in intragenic region. Remarkable variability was seen in this repeat from 13 to 20 copies when passaged through armadillo (Truman et al 2004).

- **GTA (9)**: This is a three nucleotide repeat located in intergenic region. Three human biopsies from North America exhibited alleles 9 and 10 for (GTA)9 (Truman et al 2009). A study
undertaken in Mali, south Africa reported 9 alleles of ranging from 8-15 but 9 being the most common (GTA)9.

- **(6-7):** This is a 6 basepair repeat which is translated as proline-alanine repeat. Three isolates from North America showed 7 repeats of 6-7 (Groathouse et al 2004). Predominance of 6 repeats was reported from Thailand, Brazil and Columbia (Castro et al 2009, Fontes et al 2009, Phetsukuri et al 2009) whereas Philippines reported predominance of 7 repeats (Kimura et al 2009, Sakamuri et al 2009)

- **(12-5):** This is a 12 basepair translated as Glu-Val-Val-Glu repeat. Reports show the predominance of 5 repeats in Malawian patients (Young et al 2008). Samples from Philippines and Thailand exhibited four repeats (Kimura et al 2009; Sakamuri et al 2009). Predominance of 3 repeats was observed in China (Xing et al 2009). Frequency of 5 copies was higher in Brazilian population and Columbian patients exhibited equal distribution of 4 and 5 repeats.

- **(21-3):** This is a 21 base pair repeat located in a pseudogene and predominance of 3 (82.81%) repeats have been reported from Philippines and Thailand reports 3(43.75%) and 2(56.05%) repeats. Isolates from China report 2 copies of this trinucleotide repeat (Xing et al 2009)

- **(23-3):** This is a 23bp repeat located in intergenic region and predominance of two repeats (100%) has been reported from Philippines, Thailand, China and South America (Cardona-

- **(18-8):** This is a putative uncharacterized protein/conserved membrane protein located in intragenic region. Predominance of 8 (88.13%) repeats has been reported from Philippines and South America (Cardona-Castro et al 2009, Fontes et al 2009, Sakamuri et al 2009). Predominance of 7 repeats has been reported from China (Xing et al 2009).


- **(AC)9:** Two basepair repeat in the intergenic region.

- **(AT) 15:** Di nucleotide repeat in intergenic region. This loci is known to exhibit a high degree of polymorphism as reported in various studies (Fontes et al 2009, Phetsukuri et al 2009, Xing et al 2009).

- **(AC) 8a:** It is located in intragenic region of a pseudogene similar to eukaryotic adenylate/guanylate cyclases.

- **(AC) 8b:** This is a dinucleotide repeat located in the intergenic region.

- **(GGT) 5:** This is a trinucleotide repeat located in intergenic region. Patients from China (100%), Brazil (97.64) and Columbia (100%) exhibited 4 repeats (Cardona-Castro et al 2009, Fontes
et al 2009, Xing et al 2009) those from Philippines (Kimura et al 2009, Sakamuri et al 2009) showed a predominance of 5 repeats (75%) and Thai (Phetsukuri 2009) patients exhibited equal distribution of 4 (47.91%) and 5 (48.95%) copies.

- **(TA)10**: Di-nucleotide repeat in intergenic region
- **(ML)1**: 52bp repeat in pseudogene

To summarize, it is necessary to characterize existing mini and microsatellites, VNTR and new markers for differentiation of strains from a large population or for tracing chains of transmission on a small scale.

### 2.9 Single Nucleotide Polymorphism in *M. leprae*:

A single nucleotide polymorphism (SNP) is a change or variation in a single nucleotide (A, T, G, C) of the DNA. This change occurs during DNA replication and is used as evolutionary marker. Monot et al in their study observed the SNP frequency of *M. leprae* to be 1 per 28kb indicating the well conserved and highly clonal nature of the bacteria (Monot et al 2005). For comparison, SNP frequency in *M. tuberculosis* is reported to be 1 in 3kb, 1 in 3.2 bases in *Helicobacter pylori* and 1 in 1.1kb in *Salmonella typhimurium* (Kidgell 2002, Falush et al 2003, Smith et al 1993).
**2.9.1 Geographical distribution of SNP in *M. leprae***

**Figure 2.3: Global distribution of SNP in *M. leprae***

When 175 isolates from 21 countries and 5 continents were typed for SNP’s it was observed that out of the 64 permutations only 4 occurred. These were referred to as SNP type 1 to 4 as shown in Fig 2.3 (Monot et al 2005). A correlation was found with the SNP type and the geographical region. SNP type 1 occurred predominantly in Asia, the Pacific region, and East Africa while SNP Type 2 was the rarest and was detected in South Asia, Ethiopia and Malawi. SNP Type 3 was seen in Europe North Africa and America and SNP Type 4 in West Africa and the Caribbean. Out of the 175 isolates, 30 belonged to North India and Nepal and 4 isolates belonged to South India. More recently SNP typing from archival DNA from medieval bone samples
from Europe revealed SNP type 3 (Watson et al 2009). Ancient texts describe the existence of leprosy in China, India, and Egypt in about 600 BC, and skeletal remains bearing hallmarks of the disease have been found in Egypt (Monot et al 2009).

*SNP typing may assist us in understanding important factors such as disease susceptibility, location of real loci in the bacteria, and the epidemiology of the disease.*

### 2.10 Drug Resistance

Multiple Drug Therapy (MDT) was introduced in 1983 by the government of India with an objective of controlling leprosy by 2000 (WHO 1982). Prior to MDT, Dapsone was the only drug used for treatment of leprosy. Though MDT appeared to reduce prevalence, single, double and multiple drug resistant isolates have been detected (Matsuoka et al 2000, Lee et al 2001). Drug resistance in *M. leprae* is attributable to chromosomal mutations in genes encoding drug targets. These mutations occur spontaneously as a result of errors in DNA replication. These mutants are further enriched in a population by inappropriate or inadequate drug therapy (Scollard et al 2006).

Epidemiologically Drug resistance is classified as:

i.) Primary Drug Resistance: Resistance to MDT drugs in new patients who have never received any treatment earlier.
ii.) Secondary Drug Resistance: Resistance to MDT drugs in patients who relapse after completion of fixed duration of treatment, the criteria for which is defined by WHO.

2.10.1 Molecular tools for detection of Drug Resistance:

Mouse foot-pad inoculation for detection of drug resistance was the gold standard for many years. Earlier radio-respirometry techniques like the Buddemeyer assay and BACTEC were used for *M. leprae* drug screening but these assays require a large number of viable organisms from patients, which has limited the use of these techniques (Franzblau 1989, Shetty et al 1996). The advent of molecular tools and the availability of whole genome sequence have helped in development of sensitive and rapid drug sensitivity detection methods. Recently molecular methods that detect known mutations in the target genes (*folP1*, encoding dihydropteroate synthase for Dapsone, and *rpoB* encoding the RNA polymerase β subunit for Rifampicin) and *gyrA* and *gyrB* for Ofloxacin are applied for *M. leprae*.

A heteroduplex assay has been applied to detect dapsone resistance in homogenates obtained from skin biopsies of lepromatous patients. Sulphone resistance determining region (SRDR) of *folp1* gene is amplified a universal heteroduplex generator (UHG-DDS-141), a synthetic 141-bp sulfone resistance-determining region DNA fragment that contains several base pair mismatches flanking codons that are associated with dapsone resistance more sensitive and rapid (Williams
et al 2001, Honoroe et al 2001). Another technique called Touchdown PCR (SSCP-TD-PCR) evaluates single strand confirmation polymorphisms. This technique starts with a high annealing temp and for subsequent steps decreases the annealing temp by 1°C. This reduces non-specific amplification and increases product yield. PCR product is heated to dissociate the strands are resolved on native PAGE. SSCP-TD-PCR has been used for detection of mutations in the folp1 and rpol genes (Honoroe et al 1993, Kim et al 1996, Kim et al 2003). Other studies report the use of PCR–solid-phase hybridization, allele specific PCR and Line Probe Assay for detection of mutations (Honoroe et al 2001, Sapkota et al 2006, Sapkota 2008).


### 2.10.2 Mechanisms of drug action and resistance

In 1943 at Carville Louisiana, Promin was the first sulphone chemotherapeutic agent reported for the treatment of leprosy (Faget et al 1943). Dapsone 4,4-diaminodiphenylsulfone, a synthetic sulfone
and analogue of para amino benzoic acid was introduced in 1949 (Jacobson et al 1978). It is a bactericidal sulphone which targets the folp1 gene of M. leprae. Mechanism of action of dapsone is mainly via competitive inhibition with para amino benzoic acid for the enzyme Dihydropterate synthase (DHPS), which is the main enzyme encoded by folp1, involved in folic acid synthesis. The first clinically suspected dapsone resistant case was reported in 1953 (Wolcott et al 1953). An association between dapsone resistance and mutation in folp1 has been linked to missense mutations at codon53 (ACC) or codon55 (CCC). Specifically, threonine or proline, in folp1 is known to confer dapsone resistance (Kai M et al 1999, Williams et al 2000).

Rifampicin (3(4-methly-1-piperazynl)-imino)-methly), is a bactericidal drug used for the treatment of leprosy. To date, it is the most potent drug in MDT and a single dose of 1200mg is known to be significantly bactericidal. DNA-dependent RNA polymerase, a large complex enzyme composed of four different subunits (α, β, β1 and σ encoded by rpoA, rpoB, rpoC and rpoD) is highly conserved among bacterial species. The holoenzyme binds to the upstream promoter and σ factor confers specificity. Characterization of rpoB gene in E. coli demonstrated that rifampicin specifically interacts with the β-subunit of RNA polymerase, which is required for chain initiation and extension, thereby hindering transcription. The drug is rapidly absorbed if taken on empty stomach and gets distributed throughout the body. A high bactericidal activity was shown experimentally and
clinically in 1970’s leading to the introduction of rifampicin for leprosy treatment. A correlation between rifampicin resistance and mutations in highly conserved regions of the \textit{rpoB} gene has been indicated. Isolates confirmed to be rifampicin resistant harbor mutations at codon 407, 410, 420, 425 and 427.

Clofazamine, a rimino-phenazine dye, was first used for the treatment of leprosy in the early 1960 (Browne 1985). WHO has reported the drug to be bactericidal (WHO 1982). The mechanism of action of this drug is yet to be elucidated but it is known to have inherent anti-inflammatory activity and a possible bactericidal activity through the preference of binding to a GC rich region of the bacteria (Morrison et al 1976). Molecular basis for resistance to this drug is yet to be established. Due to its anti-inflammatory activity, this drug is used to control ENL. The evaluation of the drug and determination of its minimal inhibitory concentration (MIC) in the mouse foot-pad system is difficult because of its persistence in tissues for prolonged periods of time. The compound produces a coloration of the skin leading to enhanced pigmentation. As the infiltrate diminishes, the coloration tends to diminish. Clofazamine has been used for treatment of leprosy for over 5 decades but reported drug resistance is rare.

Ofloxacin is a fluoroquinolone with moderate bactericidal activity. The principal target is DNA gyrase, a type II topoisomerase, that is composed of two A and two B subunits encoded by the genes \textit{gyrA} and \textit{gyrB} respectively. It binds to subunit A of DNA gyrase and inhibits
DNA replication. Fluoroquinolone resistance in *M. tuberculosis* is associated with point mutations within a part of *gyrA* termed the quinolone-resistance-determining region (Chopra *et al* 1997, Musser 1995). First Ofloxacin resistant *M. leprae* was reported in 1994 (Cambau *et al* 1997). Ofloxacin resistant cases reported in 2000 and 2003 were found to be associated with mutation at codon 91, GCA to GTA (Alanine to Valine) of the *gyrA* gene.

2.11 Host factors in susceptibility to Leprosy

Until the discovery of *M. leprae* by Armaeur Hansen in 1973, leprosy was considered an inherited disease (Boeck & Danielssenn 1847). It has been estimated that 0.1-1% of the population develops clinical disease suggesting the role of host immunity (Siddiqui *et al* 2001). On exposure to *M. leprae*, a strong innate immune response may help prevent the disease to establish itself in the host. On the other hand, an insufficient innate immune response may allow for an infection and thus be a determining genetic factor (Scollard *et al* 2006). Genetic factors also influence acquired immunity, which is primarily mediated through the function of T lymphocytes along with antigen presenting cells. Polymorphisms in host genes that confer genetic defects make an individual susceptible or resistant to leprosy or a particular type of leprosy. It was proposed in 1970’s that at least two different genes control the infection (de Veries *et al* 1976). Family studies, segregation analyses, and twin studies provide evidence that, in addition to
environmental and exposure factors, host genetic factors influence leprosy susceptibility (Fitness et al. 2002, Fitness et al 2004). Two approaches that have been used to study the influence of host genetics on disease are:

i) Candidate Gene Approach  ii) Genome wide scan

2.11.1 Candidate gene Approach:

The most common candidate genes studied are related to the immune system. These include human leukocyte antigen (HLA), cytokines, and other receptors.

The HLA loci are traditional candidates for association studies with leprosy with the class I HLA-A*1102-B*4006-Cw*1502 haplotype showing a highly significant association with leprosy susceptibility (Shankarkumar et al 2003). Among the class II genes the HLA-DRB1 locus, more specifically DRB1*15 and DRB1*16, are associated with leprosy susceptibility in India (de Veries et al 1980; Rani et al 1993), Thailand (Schauf et al 1985) and Brazil (Visentainer et al 1997). In contrast, the HLA-DQw1 locus was found to be associated with LL patients (Gorodezky 2004).

The gene for NRAMP1 (Natural Resistance Associated Macrophage Protein 1), is reported to be associated with susceptibility and resistance to intracellular infections in mice (Skamene et al 1998). Yet, the association of this gene with leprosy has contradictory reports (Roger et al 1997, Abel et al 1998, Hatagima et al 2001).
Toll-like receptors are cell surface molecules of innate immunity that play an important role in recognition of pathogens. Initially a Toll like receptor 2 (TLR2) was found to be associated with leprosy, but further studies indicated that this SNP (being in a pseudogene) did not encode any transcripts and the functional role could not be established (Malhotra et al 2005). More recent studies revealed an association of polymorphisms at TLR1 I602S (T1805G) and TLR1 N248S (A743G) with protection from the disease and from reversal reactions (Johnson et al 2007, Misch et al 2008, Schurring et al 2009). Minor alleles of TLR4 SNPs (896G>A [D299G] and 1196C>T [T3991I]) were found to confer protection against leprosy (Bochud et al 2009).


A SNP at the +252 position (A-G) in the first intron of the lymphotoxin (LT) -α gene together with the -308A SNP as a haplotype was also associated with protection against leprosy in a Brazilian population (Shaw et al 2001, Moraes et al 2006.) SNP in the immunomodulatory gene interleukin-10 (819 C/T) promoter has been suggested to be
associated with leprosy susceptibility in Brazil and India (Malhotra et al 2005a, Pereira et al 2009, Santos et al 2002).

The vitamin D receptor (VDR) Taq1 specific polymorphisms at exon 9 are known to be associated with leprosy in various populations. In India, the TT and CC genotypes were associated with susceptibility to lepromatous and tuberculoid leprosy respectively (Roy et al 1999, Goulart 2002, Goulart et al 2006.), whereas in Malaw., the CC genotype was associated with susceptibility to leprosy per se (Fitness et al 2004). However Nepalese population does not report any such association with leprosy (Sapkota et al 2010)

MBL (Mannose binding lectin), serum protein plays a major role in innate immune responses through complement-mediated lysis and clearance of extracellular organisms (Dommett et al 2006). Polymorphism in the MBL gene at position G161A is known to protect against lepromatous leprosy in Nepal (Sapkota et al 2010); Study from Brazil reports association of MBL gene with leprosy (de Messais reason et al 2007). Other polymorphism reported are those in Interleukin 12 receptor β1(II12Rβ1): A missense mutation at position +7809, genotype TT and TC were associated with lepromatous and tuberculoid leprosy, respectively.
One of the first studies reporting association of candidate gene in leprosy was performed on Transporter associated antigen processing molecule (TAP).

2.11.2 Genome wide association studies:

These studies have helped to reveal candidate genes conferring susceptibility to leprosy or type of leprosy. The earliest strong evidence pointed towards the major histocompatibility complex region on chromosome 6.

**Chromosome 10p13**: The first genome wide linkage scan from India mapped leprosy susceptibility to chromosome 10p13 followed by further studies indicating the association of this locus with tuberculoid leprosy (Mira et al 2003; Remus et al 2003 Siddiqui et al 2001). The MRC1 gene, encoding the human mannose receptor (MR) is located in the 10p13 region. Non-synonymous SNP’s in exon 7 of the gene have been suggested as leprosy susceptibility factors (Alter et al 2010).

**Chromosome 20p12**: This region has been linked to susceptibility to leprosy in a South Indian population (Tosh et al 2002).

**Chromosome 6q25**: This region was found to be associated with susceptibility to leprosy in Vietnam (Mira et al 2003). Further positional cloning of the gene identified a promoter region shared by PARK2 (parkin) and PARKG (parkin co-regulated gene). The haplotype
was associated with susceptibility to leprosy in Vietnam and Brazil (Mira et al 2004). An Indian study reported association of T allele at -2599 position with susceptibility to leprosy (Malhotra et al 2005).

**Chromosome 17q 11:** This chromosome has been reported to be associated with leprosy. Interestingly this region involves genes in immune regulation such as NOS2A and CCL-2, but the association of these genes with leprosy is not confirmed (Miller et al 2004; Jamieson et al 2004).

**Chromosome 8, 9, 12q12, 13q14, 16q12:** Recent genome-wide analysis of susceptibility to leprosy showed implications of genetic variants in 6 genes to be associated with leprosy in Chinese families. SNP at C13orf31 (gene encoding Chromosome 13 Open Reading Frame 31 on chromosome 13q14), two SNP’s within NOD2, (nucleotide oligomerization domain 2, chromosome 16q12), one SNP in the RIK2 gene (gene encoding receptor-interacting serine-threonine kinase 2, chromosome 16q12), and one SNP in the TNFSF15 (gene encoding Tumour Necrosis Factor superfamily member 15, chromosome 9) were found to be associated with susceptibility to leprosy. Furthermore, one SNP in LRRK2 (gene encoding leucine-rich repeat kinase) was found to be associated with MB leprosy (Zhang et al 2009).
2.12 Transporter Associated with Antigen Presentation:

The transporter associated with antigen processing (TAP), is a member of the family of ABC transporters. The main function of these ABC (ATP binding Cassette) transporters, as the name indicates is to translocate a diverse set of molecules or substances such as sugars, ions, polysaccharides, amino acids and polypeptides across the membrane (Ritz et al 2001). Each transporter is specialized for the transport of a specific substrate function and plays an important role in pathophysiologic function (Higgins et al 1992). Some of the well-characterized transporters are cystic fibrosis transmembrane conductance regulator –CFTR (Anderson et al 1991, Riordan et al 1992), a chloride channel deficient in cystic fibrosis patients. Another is the sulfonylurea receptor (SUR) 1-subunit of a potassium channel (K-ATP) in pancreatic cells, which is mutated in congenital hyperinsulinism (Meissner et al 1997, Aguilar-Bryan et al 1998). The P-glycoproteins MDR1 and MDR3, transport a series of hydrophobic drugs and phospholipids (Gottesman et al 1993, Saeki et al 1993). Finally, TAP consists of two subunits, TAP1 and TAP2, and is the only ABC transporter with a unique function in the immune system.

TAP plays a crucial role in the processing and presentation of antigen to the major histocompatibility complex (MHC) class I. It is a
heterodimer consisting of two subunits TAP1 and TAP2 made up with 8 and 7 transmembrane domains, respectively (Voss et al 1999). The N and C terminus of TAP1 and C terminus TAP2 are located in the cytosol, while the N terminus of TAP2 is located in the ER. Typically TAP efficiently transfers peptides with a length of 8-16aa, and longer peptides are transferred with a very low efficiency (Koopmann 1996). The genes TAP1 and TAP2 subunits are located in MHC II locus of chromosome 6 and each consist of 11 exons. TAP is highly conserved with a homozygosity of approximately 35% between TAP1 and TAP2 in all species (Ritz et al 2001).

2.12.1 Mechanism of Peptide Transport:

Peptide transport is a multistep process, the first step being degradation of intracellular proteins in the cytosol by constitutive and IFNγ inducible proteosomal subunits. The peptide selectivity is determined by association of TAP in an ATP independent manner which is followed by slow isomerization. Further, the TAP molecule is structurally reorganized to trigger peptide translocation across the membrane, which is ATP dependent. TAP transports peptides from the cytosol to lumen of Endoplasmic Reticulum (ER), where peptides are loaded onto a dimer of MHC I heavy chain and β-globulin. The peptide loading complex is formed along with Tapasin, oxidoreductase Erp57, calreticulin, and PD1 (Ortmann et al 1997, Spee et al 1997, Suh et al 1996). Once the peptide is loaded, MHCI dissociates itself from the
complex and crosses the cytoplasm to present the antigen to CD8+ cells (Fig)

**Fig 2.4: Mechanism of peptide transport by TAP**

![Diagram of TAP mechanism](image)

**Figure 2.4:** Schematic diagram of the mechanism of Antigen processing and presentation by TAP. This figure demonstrates the molecules involved in the TAP pathway.

### 2.12.2: Polymorphism in TAP1 and TAP 2 genes

TAP gene exhibits a limited degree of polymorphism in the coding region and hence functional implications are unclear. Four potential alleles of human TAP1 and eight alleles of TAP2 have been identified in different populations, but their influence on TAP activity and/or peptide-binding specificity varies (Reuda-Faucz et al 2000). A
functional polymorphism was found for a human TAP2 splice variant (TAP2iso) that consists of an altered C-terminus. Polymorphism in the TAP gene was first reported in 1993 (Powis et al 1993) and since then various studies have reported association with several MHC associated diseases like Multiple Sclerosis, Grave’s disease and Insulin dependent Diabetes Mellitus (Kellar-Wood 1994, Rau et al 1997, van Endert 1997). Mutations in TAP gene are known to be associated with genetic diseases such as Bare Leukocyte syndrome (BLS), a disease characterized by severe down-regulation of MHC I and MHC II antigens. Severe forms of this disease are associated with mutations in TAP2 (Gadola et al 2000). Strong associations were reported between TAP1 polymorphisms in atopy and allergic rhinitis when compared to control (Ishmail et al 1997, Kim et al 2007). However, a study conducted in Japan does not report any association with TAP1 polymorphism and allergic rhinitis (Takeuchi et al 2002). Polymorphisms in autoimmune disease such as type I diabetes and rheumatoid arthritis are associated with the TAP gene and HLA (Faustman et al 1991, Yan et al 1998). Association of TAP polymorphisms has also been reported in viral infections like dengue hemorrhagic fever, hepatitis, HIV (Kuzushita et al 1999; Liu et al 2003; Xu et al 2007; Soundravally 2007, 2008).

Some of the commonly reported polymorphisms are,
TAP1:

i) 333 (Isoleucine to Valine, ATC to GTC) on Exon 4 rsNo 1057141: A Single Nucleotide polymorphism at position of codon 333 translates Isoleucine to Valine. Iso/Val(AG genotype) has been reported to be associated with Rheumatoid arthritis, allergic rhinitis, systemic lupus erythematosus (Kim et al 2007, Foley et al 2009, Feng et al 2009)

ii) 637(Aspartic Acid to Glycine, GAC to GGC) on Exon 10 rsNo 1135216: A single nucleotide polymorphism at position of codon 637 which translates Aspartic Acid to Glycine. This has been reported to be associated with allergic rhinitis, atopic dermatitis, cystic echinococcosis (Kuvata et al 1995, kipper et al 2010)

TAP2:

i) 565 (Alanine to Threonine) G to A Exon9 rsNo 2228396: A Single Nucleotide polymorphism at position of codon 565 translates Alanine to Threonine. Ala/Thr (GA genotype) has been reported to be associated with sarcoidosis and ankylosing spondilytis (Kim et al 2007, Foley et al 1999,Feng et al 2009).

ii) 665 Threonine to Alanine: A to G Exon 11 rsNo 241447
A Single Nucleotide polymorphism at position of codon 665 translates Threonine to Alanine. Polymorphisms at the 665 position were found to be associated with cervical cancer (Ivansson et al 2008). 379 (Iso to Val, G to A)
Apart from these, other polymorphic residues of TAP1 at (codon 200, 254, 997, 648 and 661) and TAP2 (codon 436 and 651) (Feng et al 2009)

### 2.13 Purinergic Receptors:

**P2X receptors** are a family of cation-permeable ligand gated ion channels that open in response to extracellular adenosine 5'-triphosphate (ATP). They belong to a larger family of receptors known as the purinergic receptors (Grahames et al 1999, Humphreys et al 2000, Chused et al 1996) are present in a diverse array of organisms including humans, mouse, rat, rabbit, chicken, zebrafish, bullfrog, fluke, and amoeba. Purinergic receptors are plasma membrane molecules involved in several cellular functions such as vascular reactivity, apoptosis and cytokine secretion and yet only partially known (North 2002, Khakh and North 2006). These receptors have their effect of high glucose concentration on ATP-mediated responses in human fibroblasts (Khakh and North 2006). Each functional P2X receptor is a trimer, with the three protein subunits arranged around the ion-permeable pore. To date, seven separate genes coding for P2X subunits have been identified, and are referred to as P2X1 through P2X7 (North 2002, Egan et al 2006).

This receptor functions as a ligand-gated ion channel and is responsible for ATP-dependent lysis of macrophages through the
formation of membrane pores that are permeable to large molecules. Activation of this nuclear receptor by ATP in the cytoplasm may couple cellular activity with gene expression. Purinergic receptors are primarily expressed on macrophages, and microglia and to a lesser extent on astrocytes, godendrocytes and neurons and are further up regulated by IFN-γ.

2.13.1 *P2X7* Nucleotide Receptor:

P2X7 receptors are an ion-gated channel with two transmembrane domains and a trimeric structure in the plasma membrane (Rassendren et al 1997, Nicke et al 1998). Upon activation of P2X7, the cation-gated channel dilates to allow entry of larger cations (Rassendren et al 1997, Wiley et al 1998) which may lead to apoptotic death of the target cell by triggering the caspase cascade (Ferrari et al 1999, Humphrey’s et al 2000). This receptor was first described in the cells of hematopoietic system in which it played a role in cell lysis. P2X7 receptors play a dual role in neurodegenerative diseases either by optimum activation of astrocytes by assisting recovery or prolonged activation of astrocytes inhibits the multiplication of neuronal cells, release of toxic material which leads to the death of adjacent astrocytes and neurons, eventually leading to neurodegeneration (Guthrie et al 1999, Nedergaard et al 2003, Scemes et al, 2000)
2.13.2 P2X7 polymorphisms:

The human P2X7 receptor gene has 13 exons and is located on chromosome 12q40 (Buell et al. 1998). Human P2X7 receptor subunit is made up of 595 amino acids and bears a long cytosolic carboxyl terminus, which is nearly 120aa longer than the other P2X receptors (Lee et al. 2006). It is multimeric and each subunit contains two transmembrane domains connected by a large extracellular loop and intracellular N and C termini. This long carboxyl terminus is necessary for the permeability properties of the P2X7 receptor. Phosphorylation of tyrosine at amino acid 343 of P2X7 primary structure has been proposed as being important for maintaining the full activity of the P2X7 channel. P2X7 also stimulates intracellular caspases and kinases, which eventually leads to cytolysis of lymphocytes, macrophages, and dendritic cells, this also brings membrane blebbing, which is a typical morphological feature of the apoptotic process (Di Virgilio 1995, North 2002).

Studies have shown that various gene polymorphisms affect the function of P2X7 receptor which causes its association with a number of diseases. Controversial reports on P2X7 genetic polymorphisms and its association with TB in different population have been published.
These include:

**-762 T to C polymorphism**

-762 T to C SNP in the promoter region of P2X7 has been described to have a protective effect against TB in Gambian population (Li et al 2002).

**1513 A to C polymorphism**

1513 A to C polymorphism (rs3751143), because of which Glutamic acid at position 496 changes to Alanine. The function of the P2X7 receptor in macrophages from subjects homozygous for the 1513C allele is ablated. This causes impairment of cation fluxes in variety of cells, thereby leading to the release of IL-1, IL-18, Matrix Metalloproteinase (MMP)-9 from macrophages. The shedding of CD23 and CD62L from lymphocytes make the receptor non-functional but the expression of receptor is not affected, suggesting that the loss of function resulted from impaired protein-protein interactions in the P2X7 complex at the cell membrane (Gu et. al 2001, Gu and Wiley 2006).

**1729 T to A polymorphism**

P2X7 1729 (rs1653624) T to A, because of which Isoleucine changes to Aspargine at 568. This is associated with the trafficking motif at carboxyl tail of receptor. The loss of expression of the receptor makes it a marker for susceptibility to various diseases (Wiley et al 2003).
**Aim:** To study the genetics of pathogen and host in leprosy.

**Specific Objectives:**

**Pathogen:**

- *M. leprae* strain typing by VNTR and SNP to gain insight into transmission of the disease
- To study the prevalence of primary and secondary drug resistance in leprosy.

**Host:**

- To elucidate the association of TAP polymorphisms in leprosy
- To elucidate the association of P2X7 polymorphisms in leprosy
Plan of Work

Approval Institutional Ethical Committee
Newly diagnosed leprosy patients and relapse patients (n=100)
↓
Clinical Diagnosis
↓
Consent obtained
↓
Slit Skin smear & Punch Biopsy
↓
DNA Extraction

PATHOGEN

1. VNTR 18 STR loci
   Multiplex PCR
   Fragment Length Analysis

2. SNP typing
   PCR-RFLP
   Drug Resistance
   $folP1$ (dapsone)
   $rpoB$ (rifampicin)
   $gyrA, B$ (ofloxacin)
   Multiplex PCR
   Direct Sequencing

HOST

3.
4.

Controls = 100
Single Nucleotide Polymorphism

ARMS- PCR
TAP1: Iso333Valine
Asp637Gly
TAP2: Ala565Thr
Thr665Ala

PCR-RFLP
P2X7: -762 T/C
P2X7: 1513 A/C