CHAPTER ONE
INTRODUCTION

1.1 Overview on leprosy

Leprosy or Hansen’s Disease is a chronic human disease caused by *Mycobacterium leprae*. The disease has a long incubation period, usually three to five years. Leprosy is mainly a peripheral nerve disease but *M. leprae* also attacks skin and sometimes certain other tissues, like the eye, the mucosa of the upper respiratory tract, bone, muscle, and testis (Bryceson and Pfaltzgraff, 1990).

Involvement of nerve results frequently in pain and in loss of sensation, primarily of the extremities. This renders the patient vulnerable to repeated injuries, with consequent deformity and disability (WHO, 1987).

1.1.1 History of leprosy

Leprosy is often referred to as the oldest disease known to man. Leprosy is conceded by most historians to be a very ancient disease whose origins, though shrouded in the mists of antiquity, are traced back by one authority to ‘Old Stone Age’ times in Equatorial Africa (Hudson, 1964).

The earliest records which give accurate descriptions of the disease are as early as 600 BC from India (Lowe, 1942; Dharmendra, 1967). From there, true leprosy spread to China in about 500 BC and thence to Japan (Hastings, 1985). Leprosy reached the Mediterranean countries in 327 - 326 BC from India. It then spread slowly through the Greek and Roman Empires and later into Western Europe, reached epidemic proportions in the 12th - 13th century, then slowly declined. Leprosy was introduced into the Americas by Spanish and Portuguese settlers and their negro slaves, and by the French into Canada. At the present, leprosy is common in Asia, Africa, and Central and South America. In developed countries, infections with mycobacteria have been recurrently observed in patients suffering from acquired immunodeficiency syndrome and in patients under immunosuppressive therapy (Arnoldi et al., 1992).
Very little information is available on the early history of leprosy in Peninsular Malaya and Singapore until about the early 1800s, apart from some vague and scanty references to the disease by Portuguese historians of the 15th century (Lee, 1978). It is believed that in Malaysia, the disease was brought into the country by Chinese and Indian migrants in nineteenth century (Jayalakshmi, 1994).

According to Bryceson and Pfaltzgraff (1990), the factors which have in the past set leprosy apart from other diseases are:

(i) The extremely slow generation time of the bacillus. *M. leprae* takes nearly two weeks to multiply. As a result, it takes a long incubation period, a very slow development of pathology, a slow and insidious clinical evolution and an unclear epidemiological pattern.

(ii) *M. leprae* has never been grown in an artificial medium.

(iii) This is the only bacillary disease with a predilection for nerve tissue.

(iv) Only human gets leprosy, although naturally infected armadillos have been found in the southern USA, and primates in Africa.

(v) Until very recently there was no satisfactory way of detecting past or in-apparent present infection. Epidemiological studies were therefore based mainly on detection of clinical cases.

(vi) Leprosy is a disease which has a spectrum from complete absence of resistance by the host to effective immunity, which is often accompanied by extreme and destructive hypersensitivity.

(vii) Leprosy is unique in its psycho-social aspects. This is to be related to the fact that leprosy deforms and disables but seldom kills, so that those it has crippled live on, getting steadily worse, their deformities visible to all the community. The attitude of society toward those suffering from leprosy has given rise to many unfortunate incidents of insult, rejection and even murder of patients.
1.1.2 Properties of *M. leprae*

In 1873, Armauer Hansen in Norway discovered the leprosy bacillus, which appears to have been the first bacterium identified to be disease-causing in man (Feeny, 1964; Vogelsang, 1978). *M. leprae* or Hansen bacillus is an acid-fast bacillus (AFB) that has not yet been cultivated in cell-free culture media (Garbutt, 1965). Taxonomically *M. leprae* is classified under the order of Actinomycetales and the family of Mycobacteriaceae (Runyon et al., 1974). *M. leprae* is a straight or slightly curved rod-shaped organism with parallel sides and rounded ends. In infected tissue the rods are often stacked or clumped together in globi. Purified armadillo-derived *M. leprae* measured and weighed as follows: diameter = 0.25 - 0.30 μm, length = 2.1 ± 0.5 (s.d.) μm, and mass of an individual bacterium = 3.9± 1.0 (s.d.) x 10⁻¹⁴ g (Draper and Missell, 1977). Like other species of mycobacteria, *M. leprae* divides by binary fission. It is Gram-positive and strongly acid-fast following staining with carbol-fuchsir.

During logarithmic multiplication, *M. leprae* multiplies with a generation time of 10 - 15 days in the foot-pads of normal mice (Shepard and McRae, 1965a; Levy, 1976). The genome size of *M. leprae* is 2.2 x 10⁹ Da which is about 5 fg and the G+C content of the DNA is 56% (Imaeda et al., 1982; Clark-Curtiss et al., 1985). As in other members of Actinomycetales, the cell-wall of *M. leprae* contains mycolic acids (high molecular weight, branched-chain hydroxy-fatty acids), arabinogalactan, and peptidoglycan. Mycolic acid is responsible for the characteristic acid-fastness seen when the organism is stained with carbol-fuchsir.

*M. leprae* contains only about 1,000 protein-coding genes compared to the estimated 4,000 present in *E. coli* (Cole, 1994). *M. leprae* metabolizes carbon sources through the classical pathway of glycolysis, the hexose monophosphate shunt and tricarboxylic acid cycles (Wheeler, 1983). Unlike other mycobacteria, *M. leprae* may not synthesize purine bases of nucleotides in order to make nucleic acids and for oxidative metabolism, and may have to scavenge them from the host cell (Khanolkar et al., 1978; Khanolkar and Wheeler, 1983). Mycobacteria also need iron which they extract and take up from the host by chelation with mycobactins. *M. leprae* lacks mycobactin. It is conceivable that the gene sparsity and the metabolic defects such as these may perhaps explain why the organism has proved so difficult to culture *in vitro* and its slow growth.
In a laboratory, *M. leprae* may be identified by the following methods:

(i) It is acid-fast when stained with carbol-fuchsin and this acid-fastness may be removed or 'extracted' by pre-treatment with pyridine.

(ii) It will not multiply in conventional media that support the growth of other mycobacteria.

(iii) It will multiply in the foot-pads of mice, especially immunodeficient mice.

(iv) It contains a unique glycolipid (phenolic glycolipid I, PGL-I) and arabinomannan (lipoarabinomannan B, LAM-B), which can be identified serologically.

(v) Its deoxyribonucleic acid (DNA) may be extracted and shown to be homologous with that of established isolates of *M. leprae*.

Therefore, inoculation of *M. leprae* in foot-pads of normal mice (Shepard, 1960), and its development in immunodeficient mice, where growth of *M. leprae* is more extensive (Rees, 1966), are the model infections that have contributed most to leprosy bacteriology. On the other hand, the discovery by Kirchheimer and Storrs (1971) that the nine-banded armadillo is naturally susceptible to leprosy, with resulting heavy and systemic infection, has greatly increased the supply of *M. leprae* for basic research.

1.1.3 Classifications of leprosy

Infection with *M. leprae* is usually considered to take place through the skin, or through the nasal mucosa from droplet infection. Most people who are infected with *M. leprae* develop a subclinical infection which they will recover naturally without ever having symptoms or signs of disease. Only few individuals develop the disease, leprosy. The organism multiplies best in the cooler parts of the body, so that the skin of the face, limbs and the more superficial nerves are preferentially invaded. The bacillus multiplies inside macrophages, both those of the skin (histiocytes) and, especially of the nerves (Schwann cells). This usually elicits an inflammatory response of histiocytes and lymphocytes. Clinically there appears a small vague macula which is hypopigmented in a dark skin and erythematous in a light skin. The lesion is called indeterminate as there is no indication how it will develop. Over 70% of indeterminate lesions heal spontaneously. If bacillary growth outstrips the defence mechanisms, then the condition progresses into one of the patterns that make up the
spectrum of disease in leprosy, which depends upon the degree to which cell mediated immunity (CMI) develops or is suppressed and the extent of bacillary multiplication (Figure 1).

In 1966, Ridley and Jopling defined five groups or classes of leprosy referred to as full tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL) and full lepromatous (LL) on the basis of clinical, bacteriological, histological, and immunological characteristics. The definitions were primarily based on the estimation of the number of bacilli in biopsy sections (Figure 2). The borderline group in the Ridley-Jopling classification represents a wide spectrum between the two polar types – typical tuberculoid at one end (the resistant end) and typical lepromatous at the other end (the non-resistant end).

1.1.4 The differential diagnosis of leprosy

According to the World Health Organization (WHO), 1988, the diagnosis of leprosy was based on the demonstration of at least two of the following cardinal signs:

(i) a characteristic skin lesion. The essential characteristic of lesion of tuberculoid leprosy in a dark skin is hypopigmentation, whether the lesion is macular or infiltrated. In a light skin the lesion is copper coloured or red.

(ii) sensory loss or anaesthesia. This may be of individual skin lesions or in the distribution of a large peripheral nerve, as in tuberculoid leprosy; or it may be over the areas of fine nerve involvement in lepromatous leprosy, beginning with the extensor surfaces of the forearms and legs, hands and feet.

(iii) thickened nerves. Sites of predilection are, in order of frequency: the ulnar nerve immediately above the olecranon groove, the posterior tibial nerve behind the internal malleolus, the common peroneal (lateral popliteal) nerve in the popliteal fossa and proximal to where it winds round the neck of the fibula, the radial cutaneous nerve at the wrist, the facial and great auricular nerves, and the median nerve proximal to the flexor retinaculum.

or (iv) on the presence of acid-fast bacilli (AFB) in slit-skin smear. If the diagnosis is still in doubt, then a skin biopsy of the lesion should be taken for histopathological study.
Figure 1: The course after infection with *M. leprae*.
Adapted from Hastings (1985)
The density of bacilli is recorded as the bacteriological index:

BI 6+ – Many clumps or over 1,000 bacilli in an average microscopic field
BI 5+ – 100 to 1,000 bacilli in an average microscopic field
BI 4+ – 10 to 100 bacilli in an average microscopic field
BI 3+ – 1 to 10 bacilli in an average microscopic field
BI 2+ – 1 to 10 bacilli in 10 microscopic fields
BI 1+ – 1 to 10 bacilli in 100 microscopic fields
BI 0+ – 0 bacillus in an 100 microscopic fields

Figure 2: The five groups of leprosy with their bacteriological index (BI) and cell-mediated immunity (CMI) response.
Modified from Bryceson and Pfaltzgraff (1990)
Three of the above-mentioned are based on clinical examinations. Laboratory investigation concerned with the bacteriological aspect of the patient with leprosy, whether for diagnosis, classification, or monitoring response to treatment, depends on the demonstration of slit-skin smear technique, as *M. leprae* cannot be cultured.

1.1.4.1 Slit-skin smear

In this technique, seven smears are made from suspect lesions as well as from sites commonly affected in leprosy patients, usually the earlobes, forehead, chin, extensor surfaces of forearms, dorsal surface of fingers, buttocks, and trunk. Thus, from the smears, the estimation for the number of *M. leprae* (bacteriological index, BI) and the proportion of viable bacteria (morphological index, MI) are available.

The measurement of the BI is carried out under oil immersion (objective x100, eyepiece x6 or x8) by determining the number of AFB/field after examining 25 - 100 fields, on a logarithmic scale ranging from 0 - 6 (Ridley, 1958). In particular, this index extends from the presence of very few to very many AFB, and the steps (1+, 2+, etc.) are regularly spaced, each additional unit indicating a ten-fold increase in the number of AFB (Figure 2). All AFB are counted whether stained uniformly or irregularly, and the scores of the smears from a number of different skin sites added together and divided by the number of sites is the BI of the patient.

The BI is high in untreated patients with lepromatous and borderline lepromatous leprosy, and low in patients with tuberculoid leprosy (Figure 2). During effective treatment of multibacillary leprosy, the BI decreases, at a rate of about 90% per year for lepromatous patients, and perhaps more rapidly for borderline lepromatous patients (Shepherd *et al.*, 1968).

An important information from the stained slit-skin smears used to measure the BI is the MI. MI is the percentage of solid staining bacilli representing live bacilli picked randomly from the smear. At least 100 organisms were counted, when available. Only individual bacteria, the entire outline of which can be seen, and which are not touching or superimposed, are counted. Bacilli that stain irregularly or are beaded or fragmented are dead and will be excluded (Waters and Rees, 1962). Unfortunately, the MI reading varies widely from laboratory to laboratory (Lombardi *et al.*, 1994). Thus MI may overestimate the proportion of viable *M. leprae* as it is not known what interval elapses between biological death and the degenerate changes
resulting in the irregular staining of *M. leprae* (Shepard and McRae, 1965b; McRae and Shepard, 1971).

### 1.1.4.2 Skin biopsy

Although the histopathological interpretation of skin biopsy helps in the diagnosis of leprosy (Bryceson and Pfaltzgraff, 1990), the skin biopsy has another important role in animal inoculation especially for mouse foot-pad (MFP) assay. The MFP assay is particularly used to monitor the emergence of drug resistant strains of *M. leprae* by leprosy control services (WHO, 1987). Besides, from the skin biopsy homogenate (used in MFP inoculation), the microscopic density count and viability count of *M. leprae* in the biopsy can be acquired by acid-fast staining. The microscopic density count and viability count represent the bacterial load and the live bacilli in a single skin biopsy, in contrast to BI and MI which represent the bacterial load and live bacilli from seven smears made from suspect lesions and common sites in leprosy patients. Therefore, the microscopic density count and viability count give information on a specific site.

### 1.1.5 Treatment and control of leprosy

Until 1941 there was no really effective antileprosy drug, although hydnocarpus (chaulmoogra) oil, which had been used in India and China for centuries, was of limited value (Schujman, 1947; Joshua-Raghavar, 1983). Sulphones were first used in treating leprosy in 1941, and still remain the most useful drug. Diaminodiphenyl sulphone (dapsone, DDS) is bacteriostatic but its exact mode of action is not known. It is a competitive inhibitor of para-aminobenzoic acid and interferes with folate metabolism, but the unique sensitivity of *M. leprae* to DDS suggests that some other mechanisms may also be involved (Hastings, 1977). In 1983, Kulkarni and Seydel showed that the high sensitivity of *M. leprae* to DDS was entirely attributable to the high affinity of its folate synthetase for DDS. The MI of bacilli from smears of patients with lepromatous leprosy treated with DDS falls to zero in five to eight months. The first trial with DDS in Malaysia was carried out in the Sungai Buluh leprosarium (National Leprosy Control Centre, NLCC, S.B.) in 1948. In 1964, the earliest DDS-resistant cases of leprosy were detected in three patients in NLCC, S.B., by the MFP assay (Rajagopalan, 1983). Since then, DDS resistance has become an increasing problem around the world.
Rifampicin (Rifadin) is an effective anti-leprosy drug which brings down the MI in lepromatous leprosy to zero in about five weeks. Rifampicin acts by inhibiting the DNA-dependent RNA polymerase of microorganisms thus interfering with bacterial RNA synthesis (Hastings, 1985). Unfortunately it is very expensive, may produce toxic syndromes and persistent viable bacilli can still be found in preferred sites in lepromatous patients who have been treated with rifampicin daily for 5 years (Jacobson and Hastings, 1976).

Streptomycin is bacteriostatic against *M. leprae* but it has now given way to rifampicin. It is given by intramuscular injection. When used on its own, resistance develops rapidly. Clofazimine has an action equal to that of DDS and also an anti-inflammatory effect. The drawbacks of clofazimine are its cost, the development of skin pigmentation and abdominal symptoms. Persistent viable bacilli have been found in lepromatous patients treated for 6 years with clofazimine (Warndorff-van Diepen, 1982). Ethionamide and prothionamide are more expensive and more toxic than DDS. Resistance develops within a few years in some patients when ethionamide or prothionamide is given as monotherapy (Pattyn and Colston, 1978). Hepatotoxicity seems to be more of a problem when the drugs are administered with rifampicin. Owing to their toxicity, thionamide drugs are the least desirable bactericidal drugs for oral use.

In 1982, the WHO drew up specific recommendations for multiple drug therapy (MDT) to treat leprosy with more than one drug in order to prevent an epidemic of DDS resistance and to boost the success rate of leprosy control programs. The drugs used in MDT include rifampicin, DDS and clofazimine. The benefits of MDT are to prevent drug resistance; eliminate the need to determine the sensitivity of *M. leprae* to the drugs before starting treatment; shift the concept of prolonged treatment that merely arrests the disease to a short course of treatment that cures the disease; prevent deformity more efficiently and reduce the long-term costs of control program (Bryceson and Pfaltzgraff, 1990). With the implementation of MDT in 1985 in Malaysia, the national prevalence rate of leprosy has reduced from 5.7 per 10,000 in 1983 to 1.7 per 10,000 in 1992 (Jayalakshmi, 1994).

1.2 The polymerase chain reaction (PCR)

Mullis *et al.* (1986) discovered PCR as an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the target DNA to be amplified and
repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase (Innis et al., 1990). The result is an exponential accumulation of the specific target segment, approximately $2^n$, where $n$ is the number of cycles of amplification performed.

Synthetic oligonucleotides are used as primers to initiate polymerization of a specific region on each of the single strands, framing a DNA segment for amplification. DNA polymerase I is used in the amplification process after the double-stranded DNA is separated by heat denaturation. In the presence of the four deoxyribonucleoside triphosphates, polymerization of complementary strands of DNA is initiated. Each phase of the reaction cycle is controlled by temperature adjustments and repeated cycles of heat denaturation for DNA strand separation lead to rapid destruction of the DNA polymerase activity necessitating addition of fresh enzyme at each cycle. Use of a thermostable DNA polymerase, *Taq* DNA polymerase isolated from *Thermus aquaticus*, alleviated this problem and allowed for automation of the reaction making the process practical for research laboratories (Gillis, 1991).

The first DNA sequence to be amplified by PCR was the human β-globin gene (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987). Later, PCR has been used in the examination of nucleotide sequence variations (Saiki et al., 1986; Bos et al., 1987; Embury et al., 1987) and chromosomal rearrangements (Lee et al., 1987). PCR is also used for direct sequencing of mitochondrial (Wrischnik et al., 1987) and genomic DNAs (McMahon et al., 1987; Wong et al., 1987), and for the detection of viral pathogens (Kwok et al., 1987).

Today many different types of PCR have been developed. Asymmetric PCR has been developed to amplify one strand far more than the other by adjusting primer concentrations in favour of one DNA strand. After 10 to 15 cycles one of the primers is used up and only the strand complementary to the other primer continues to be copied (Gyllensten and Erlich, 1988).

Inverse PCR amplifies the stretches of unknown DNA on either side of the known sequence. This is accomplished by circularizing a piece of DNA and hybridizing primers to the ends of the known sequences in an orientation opposite the customary one. This results in the amplification of the primer-flanking regions rather than the regions flanked by the primers (Ochman et al., 1988; Triglia et al., 1988).
Anchored PCR can be employed to generate multiple copies of a desired DNA segment when only one end of a sequence is known. To the unknown end is covalently attached a segment of guanines which can serve as template to a polycytosine primer. Together with the primer which recognizes the known sequences on the opposite end of the DNA, amplification can occur normally (Frohman et al., 1988; Loh et al., 1989).

Nested PCR has been applied to enhance the sensitivity and specificity of PCR by subjecting the PCR product to a second round of PCR with an internal set of primers. The nested strategy has an advantage because of the addition of the fresh reagents and the greater number of cycles. Furthermore, dilution of amplified products reduces the inhibitory factors present. However, nested PCR involves extra manipulations, extra cost, and is more prone to contamination (Plikaytis et al., 1990; Pierre et al., 1991). There are other nucleic acid amplification methods including the use of enzymes other than DNA polymerase such as T7 RNA polymerase in transcription-based amplification systems (Kwoh et al., 1989).

Unquestionably, no single protocol will be appropriate to all applications. Consequently, each new PCR application would require optimization.

1.3 Application of PCR in leprosy

Early diagnosis of leprosy is essential for effective chemotherapy in leprosy control program. However, at the moment, there is still no available method that is specific and sensitive enough to confirm the diagnosis of leprosy at a very early stage before the appearance of clinical signs (subclinically infected cases) or even at a later stage with a suspicious lesion of indeterminate leprosy.

Presently, the diagnosis of leprosy is still based on the clinical examination of a patient's lesions, demonstration of AFB in slit-skin smears, or histopathology study of a skin biopsy. These methods can be inconclusive in early disease or in the indeterminate forms of leprosy as the development of lesion or histopathology evidence may not be clear cut and the AFB are difficult to find (Talhari, 1996). Besides, the microscopic examination of smears for acid-fast staining bacilli (AFB) is not sensitive and specific. Slit-skin smears can only detect AFB present at a concentration greater than $10^4$ per g of skin, and so are negative in TT leprosy (Shepard and McRae, 1968; Bryceson and Pfaltzgraff, 1990). Often, the low density of AFB in skin has caused an underestimation of the AFB or led the AFB to be missed entirely by conventional microscopy, thus cases are missed or misclassified
(Lucas and Ridley, 1989). Besides, smears do not fully represent the bacterial density of a lesion, especially when the size of the granuloma within the lesion is small (Ridley and Hilson, 1967). The AFB may, however, be found in other mycobacterial diseases such as tuberculosis, *Mycobacterium ulcerans* infections, and *Mycobacterium marinum* infections (Hastings, 1985; Sharma et al., 1995).

Although the mouse model (MFP assay) has found great application for determining whether the causative organism, *M. leprae*, is viable and its susceptibility to anti-leprosy drugs, this technique is relatively insensitive, requiring 6 to 12 months, skilled technician, and sophisticated facilities for detecting viable organisms (WHO, 1987).

Recently, immunologic studies have defined antigenic markers suitable for reliable identification of *M. leprae* (Hunter and Brennan, 1981; Cho et al., 1983; Sinha et al., 1983; Fujiwara 1984; Engers, 1985; Khanolkar et al., 1989). These tests show a high degree of specificity but are not readily adaptable to detect small numbers of *M. leprae* in biological tissues (Gupte, 1996).

PCR can be extremely sensitive and potentially highly specific if the sequences which are selected to prime the reaction are themselves specific for *M. leprae*. The method depends on detection of DNA which is more susceptible to degradation on cell death than other cell components, so should be a more accurate indicator of viability (De Wit et al., 1991; Jamil et al., 1993). During the last decade, various gene sequences of *M. leprae* have been identified as targets for probes and PCR. These include DNA sequences that encode major antigens, such as the 15-kDa (LSR/A15 gene) (Misra et al., 1995), 18-kDa (Booth et al., 1988; Williams et al., 1990, 1992), 36-kDa (pra gene) (Hartskeerl et al., 1989; De Wit et al., 1991; Klatser et al., 1991; Jamil et al., 1993), 65-kDa (groE-L gene) (Hartskeerl et al., 1989; Hackel et al., 1990; Plikaytis et al., 1990; Li and Wu, 1995) proteins, or non-antigen-encoding sequences such as *M. leprae*-specific 16S ribosomal RNA (16S rRNA gene) (Estrada-G et al., 1988; Cox et al., 1991; Arnoldi et al., 1992; Pattyn et al., 1992) and repetitive sequence (RLEP) (Woods and Cole, 1989; Yoon et al., 1993; Jamil et al., 1994).

The PCR amplifications of the protein-encoding genes and 16S rRNA gene are specific and have the detection limit of approximately 100 bacilli in purified *M. leprae* suspensions. These methods are much more sensitive than other methods of detecting *M. leprae*, such as microscopic visualization (10⁵ - 10⁶ bacteria) (Yaeger et al., 1966).
and DNA hybridization (10^3 - 10^4 bacteria) (Shoemaker et al., 1985; Clark-Curtiss, 1988; Eisenach et al., 1988; Clark-Curtiss and Docherty, 1989).

Most of the protein-encoding genes exist as single copy in the *M. leprae* genome, such as the 18-kDa (Williams et al., 1990), 36-kDa (Thole et al., 1990; Jamil et al., 1993), and 65-kDa (Woods and Cole, 1989) proteins. In addition, some of the encoded proteins are heat-shock proteins, i.e., the 18-kDa (Nerland et al., 1988) and 65-kDa proteins (Shinnick et al., 1988) and the gene encoding for the 16S rRNA found in slow-growing mycobacterium and *M. leprae* has only a single copy in the genome (Bercovier et al., 1986; Pattyn et al., 1992). Thus, the sensitivity of the PCR may be further increased by amplification of repetitive sequence (RLEP) present in the genome (Clark-Curtiss and Docherty, 1989; Woods and Cole, 1989). This sequence has been reported to be specific for *M. leprae* by hybridization (Clark-Curtiss and Docherty, 1989), and Woods and Cole (1989) have shown the RLEP sequence to be 15-fold more sensitive after PCR than a single copy sequence.

The RLEP sequence presents in about 28 copies in the genome of *M. leprae* (Clark-Curtiss and Docherty, 1989; Grosskinsky et al., 1989; Woods and Cole, 1990). A copy of this sequence is found downstream of the *groE-L* gene (Grosskinsky et al., 1989). Nucleotide sequence analysis showed that the repetitive element RLEP consists of a 545 bp central domain flanked by a 100 bp left end and a 44 bp right end, that may be associated with a 47 bp extension (Woods and Cole, 1990). The RLEP element has few features in common with the classical bacterial insertion sequences (Jamil et al., 1994), and does not appear to code for any proteins. Thus, primers were chosen to amplify a 372 bp and later a 447 bp segment of the conserved 545 bp central domain and the limit of detection is increased to 1 - 10 bacilli (Woods and Cole, 1991).

The sensitivity and specificity of PCR amplification was then further improved by another method called one-tube nested (OTN) PCR of the RLEP sequence, followed by the detection of the amplified products by a colorimetric method. This colorimetric OTN PCR has been reported to have a sensitivity of detecting 1 fg of purified *M. leprae* genomic DNA which is less than one genome equivalent (Jamil et al., 1994).

The OTN PCR technique has been described by Kemp et al. (1990). It circumvents the requirement for opening tubes to add fresh reagents and thereby reduces the risks of contamination; moreover, there is only one reaction so it involves
fewer manipulation and is more cost-effective. Usually, PCR products are detected by agarose gel electrophoresis, which is simple but is not suited for the analysis of large numbers of samples or for automation. Alternative colorimetric detection methodologies have been developed to detect PCR products (Kemp et al., 1990; Lundeberg et al., 1990; Wilson et al., 1993). Colorimetric OTN PCR technique is suitable for routine diagnosis as it is sensitive, reliable, and amenable to large-scale screening of samples.

In the colorimetric OTN PCR by Jamil et al. (1994), four primers (two long outer primers and two short inner primers) are used to prime the different positions of the RLEP sequence. The amplification generates two major fragments of 455 bp (by the two outer primers) and 320 bp (by the two inner primers). Besides, two minor PCR products of 412 and 363 bp are produced by extension of the outer primers to inner primers (Figure 3). As a portion of the short inner primers are labelled with digoxigenin or biotin at their 5' ends, OTN PCR therefore produces 320 bp fragments which are labelled with a different ligand of each end. These labelled molecules are captured in avidin-coated microtiter plate, and detected by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by color development in the presence of para-nitrophenyl phosphate (Figure 4).

1.4 Leprosy in Malaysia

In Malaysia, leprosy is still a public health problem with an average national prevalence of 1.7 per 10,000 of the population in 1992 (Jayalakshmi, 1994). With appearance of drug-resistant *M. leprae* strains and presence of illegal immigrants, there is a trend of increasing leprosy cases detected. In the 1994 National Leprosy Control Centre Annual Report, 62 cases were reported, showing an increase of 68 and 3% over the numbers of cases reported for 1992 and 1993, respectively (Table 1).

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The national statistics also show an increasing trend of leprosy among the Orang Asli (Aborigines) of West Malaysia (Gan, 1993; NLCC Annual Report, 1994).
Figure 3: Diagrammatic representation of the positions of different RLEP primer sets. MLO1 & MLO2 are outer primers used to generate 455 bp fragment, and MLI1D & MLI2B or MLI3 & MLI4 are inner primers which give 320 bp of amplified product. Modified from Jamil et al. (1994)
(i) First phase  – high annealing temperature
   – outer primers generate long PCR product

(ii) Second phase  – low annealing temperature
   – inner labelled primers generate shorter labelled PCR

(iii) Colorimetric detection of OTN PCR product

Figure 4: Illustration of the one-tube nested (OTN) PCR and colorimetric detection protocols.
B, biotin; D, digoxigenin; A, avidin; Ab/Ap, anti-digoxigenin antibody-alkaline phosphatase conjugate.
Modified from Wilson et al. (1993)
1.5 Objectives of this study

Woods and Cole (1989) and Jamil et al. (1994) have shown that the RLEP sequence is the best choice used in PCR for direct detection of *M. leprae*. In Woods and Cole (1989), the results were based on mouse foot-pad and human biopsy samples known to be infected with *M. leprae*. The PCR results were not compared with microscopic reading (such as BI and MI). Furthermore, the application of PCR at the clinical level and field work was not done.

Jamil et al. (1994) compared the results of colorimetric OTN PCR with the BI reading of clinical samples, but not with the MI reading of clinical samples. Furthermore, mouse foot-pad samples were not tested and the use of colorimetric OTN PCR in field work was not assessed.

In addition, the investigation of the PCR applied to different patient populations from diverse geographical areas is essential to confirm its worldwide use in routine diagnosis.

This study was undertaken with the following objectives:

a) Comparison of the extraction of *M. leprae* genomic DNA between the methods of Jamil et al. (1994) and Woods and Cole (1989).


c) Sensitivity of the PCR amplification assay by the methods of Woods and Cole (1991) and Jamil et al. (1994) on purified *M. leprae* genomic DNA.

d) Determination of the efficiency of the PCR methods of Woods and Cole (1991) and Jamil et al. (1994), on both human skin punch biopsy (HSPB) samples and mouse foot-pad homogenates (MFPHs).

e) Comparison of the results from both PCR methods, by Woods and Cole (1991) and Jamil et al. (1994), with the BI, MI readings from slit-skin smear; microscopic density count and viability count of human skin punch biopsy homogenates (HSPBHs).

f) Comparison of the results from both PCR methods, by Woods and Cole (1991) and Jamil et al. (1994), with microscopic count on MFPHs.
g) Determination of the effect of storage period and storage buffer of the HSPB samples on both PCR methods, by Woods and Cole (1991) and Jamil et al. (1994).

h) Application of the colorimetric OTN PCR (Jamil et al., 1994) on clinical samples (HSPB), as well as the samples from the household contacts of leprosy patients (from a field work carried out in Sabah and Sarawak).