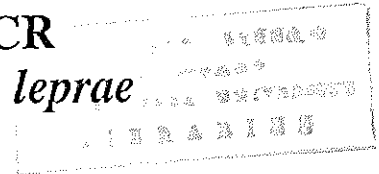


**DEVELOPMENT OF A COLORIMETRIC  
MICROTITER PLATE HYBRIDIZATION**

**ASSAY TO DETECT RT/PCR  
PRODUCTS OF *Mycobacterium leprae***

**Yoseph Haile  
June 1997**

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*A thesis submitted to the School of Graduate Studies, Addis Ababa  
University in partial fulfillment of the requirements for  
the degree of Master of Science in Biology*

**Yoseph Haile  
June 1997**

**Dedication**

**To my mother Bekelech Kidane**

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## TABLE OF CONTENTS

	PAGE
i. Acknowledgements	i
ii. List of Figures	vi
iii. List of Tables	vii
iv. Abbreviations	viii
v. Abstract	1
1. INTRODUCTION	1
1.1. The disease	1
1.2 History of the disease	1
1.3 Prevalence and distribution	2
1.4 Leprosy in Ethiopia	3
1.5 Biology of <i>M. leprae</i>	4
1.6 Mode of transmission	5
1.7 Spectrum of leprosy	6
1.8 Detection of <i>M. leprae</i>	8
1.8.1. Clinical examination of leprosy	8
1.8.2. Microscopic demonstration of Acid Fast Bacilli	9
1.8.3. Cultivation of <i>M. leprae</i> in the mouse foot pad	11
1.8.4. Lepromin test	12

1.8.5. Antibody-based detection of <i>M. leprae</i>	13
1.8.6. Antigen-based detection of <i>M. leprae</i>	14
1.8.7 (A). Molecular biology of <i>M. leprae</i>	15
1.8.7 (B). Nucleic acid-based detection of <i>M. leprae</i>	17
1.9. Objectives of this project	20
MATERIALS AND METHODS	21
2.1. RNA extraction	21
2.2. cDNA synthesis	22
2.3. Primers and probes	23
2.4. PCR amplification	26
2.5. Detection of RT/PCR products	26
2.5.1 Agarose gel electrophoresis	26
2.5.2. Southern hybridization and detection	27
2.5.3. Probe labelling for ECL	28
2.6. ELISA-based detection of RT/PCR products	28
2.6.1. Direct microtiter plate assay	28
A. Direct colorimetric microtiter plate assay	28
B. Selection of blocking reagent	29
2.6.2. Indirect assay with blocking reagent	31
A. Indirect assay with blocking reagent	31
B. Selection of coating buffer	32
C. Probe labelling with digoxigenin	32
2.6.3. Indirect assay without blocking reagent	33

A. Indirect assay without blocking reagent	33
B. Selection of probe and further optimization of incubation temperature, time, and washing buffer for indirect ELISA without blocking	34
2.7. Sensitivity of ELISA-based detection of RT/PCR products	35
2.7.1. Separation and counting of <i>M. leprae</i> from Mouse foot Pad and armadillo liver	35
2.7.2. Sensitivity testing	35
2.8. Detection of RNA in tissue specimens using GAPDH primers	36
2.9. Detection of <i>M. leprae</i> in patients biopsies	36
3. RESULTS	37
3.1. RNA extraction and selection of primers	37
3.2. PCR efficacy of biotinylated and non biotinylated primers	39
3.3. Selection of blocking reagents for direct assay	41
3.4. Selection of coating buffer	43
3.5 Comparison of different types of ELISA-based detection for RT/PCR products	45
3.6. Selection of probes	47
3.7. Further optimization of temperature, washing buffer and incubation time	49
3.8. Assesment of sensitivity	50
3.8.1. Assesment of sensitivity using serially diluted RT/PCR products	50

3.8.2. Assesment of sensitivity using serially diluted bacilli	50
3.9. Assesment of <i>M. leprae</i> in the control skin biopsies	53
3.10. Comparison of microtiter plate hybridization assay with Southern blot	57
3.11. Detection of <i>M. leprae</i> in clinical specimens	59
4. Discussion	64
5. Conclusions and recommendations	70
6. REFERENCES	71

## List of Figures

Fig. 1.	Partial nucleic acid sequence of <i>M. leprae</i> 16S rRNA.	25
Fig. 2.	Comparison of P1 and P2 sense primers.	38
Fig. 3.	Comparison of biotinylated and non-biotinylated primers.	40
Fig. 4.	Comparison of streptavidin coating buffers.	44
Fig. 5.	Comparisons of different microtiter assay.	46
Fig. 6.	Probe selection.	48
Fig. 7.	Sensitivity of the colorimetric microtiter plate hybridization assay tested with serially siluted RT/PCR product.	51
Fig. 8.	Sensitivity of colorimetric assay in detecting serially diluted bacilli.	52
Fig. 9.	RT/PCR amplification of GAPDH fragment from skin biopsies of non-leprosy patients and healthy individuals.	54
Fig. 10.	RT/PCR for <i>M. leprae</i> in control skin biopsies.	55
Fig. 11.	ELISA-based RT/PCR products to non-leprosy patients and normal individuals.	56
Fig. 12.	RT/PCR amplification of GAPDH from patients biopsies.	60
Fig. 13.	RT/PCR for <i>M. leprae</i> 16S rRNA using Bio-P2 & P3 primers after 40 cycles.	61

## Lists of Tables

Table 1.	Primers and probes used in this study.	24
Table 2.	Blocking reagents used in this study.	30
Table 3.	Comparison of different blocking reagents for direct and indirect colorimetric microtiter plate assays.	42
Table 4.	Comparison of the sensitivity of the colorimetric assay with gel-electrophoresis, and Southern hybridization of <i>M. leprae</i> 16S rRNA RT/PCR product.	58
Table 5.	Detection of <i>M. leprae</i> by ELISA-based assay for RT/PCR product.	62
Table 6.	Comparison between <i>M. leprae</i> RT/PCR and acid fast staining of specimens from leprosy patients and controls.	63

## Abbreviations

AFB	= Acid fast bacilli
AMV RT	= Avian Myeloblastosis Virus Reverse Transcriptase
BP	= Base pair
BB	= Borderline leprosy
BI	= Bacillary index
BL	= Borderline lepromatous leprosy
BT	= Borderline tuberculoid leprosy
BSA	= Bovine serum albumin
CMI	= Cell mediated immunity
cDNA	= Complimentary DNA
DNA	= Deoxribonucleic acid
DTH	= Delayed type hypersensitivity
dNTP	= Deoxynucleotide triphosphate
DTT	= Dithiothreitol
DEPC	= Diethylpyro carbonate
DIG	= Digoxigenin
ECL	= Enhanced chemiluminescence
EDTA	= Ethylenediaminetetraacetic acid
ELISA	= Enzyme linked immunosorbent assay
ENL	= Erythema nodusum leprosum
FITC	= Fluorescein
GAPDH	= Glyceraldehyde phosphate dehydrogenase

I	= Indeterminate
LAM	= Lepoarabinomannan
LL	= Lepromatous leprosy
KD	= Kilodalton
HRP	= Horseradish peroxidase
MoAb	= Monoclonal antibody
MDT	= Multidrug therapy
MI	= Morphological index
MB	= Multibacillary leprosy
OD	= Optical density
PB	= Paucibacillary leprosy
PGL-1	= Phenolic glycolipid-1
PCR	= Polymerase chain reaction
pNPP	= p Nitro phenol phosphate
Pra	= Prolin-rich antigen
RLEP	= Repetitive leprosy sequence
RT/PCR	= Reverse transcription/PCR
RNA	= Ribonucleic acid
rRNA	= Ribosomal RNA
SDS	= Sodium dodecyl sulphate
TBE	= Tris borate EDTA
Taq	= <i>Thermophilus aquaticus</i>
TT	= Tuberculoid leprosy

## Abstract

In order to improve detection of *Mycobacterium leprae* in clinical specimens, an ELISA-based detection method for a 176 bp fragment generated by RT/PCR for the 16S rRNA was developed. Total extracted RNA was reverse transcribed to make cDNA, and was subjected to PCR amplification using one biotinylated primer. The products of PCR amplification were added to streptavidin-coated microtiter plates, denatured with NaOH, hybridized with a FITC labelled 16S rRNA oligonucleotide probe, then anti-FITC antibody conjugated with alkaline phosphatase was added, and finally subjected to colorimetric detection using pNPP as a substrate to the alkaline phosphatase. Three types of microtiter plate assays were investigated. Of these, one (indirect assay without blocking reagent) was confirmed to be simple, as well as sensitive for detecting *M. leprae* in leprosy patients. This assay was highly specific as, no amplification was observed with skin biopsies from normal individuals or patients with skin diseases other than leprosy. After optimization of the assay, the sensitivity of the system was further assessed by using serially-diluted bacilli. It was very sensitive and detected as few as 10 bacilli. By examining 61 tissue biopsies from leprosy patients, the assays sensitivity for clinical specimens was assessed. The assay detected *M. leprae* RT/PCR products in 100% multibacillary patients, and in 80% of paucibacillary patients. In addition, since 16S rRNA is rapidly degraded in dead cells, application of ELISA-based RT/PCR would be most appropriate for the diagnosis of difficult cases harboring live bacteria. Such information would be important for further patient management.

## 1. INTRODUCTION

### 1.1. The disease

Leprosy is a chronic inflammatory disease of man caused by the intracellular bacillus *Mycobacterium leprae*, which primarily invades the peripheral nerves and the skin. *M. leprae* can also be found in large numbers elsewhere, particularly in the mucosa of the mouth, upper respiratory tract, reticuloendothelial system, smooth and striated muscle, liver, spleen, lymph node, eyes, blood vessel walls, bone marrow, and testes. The survival and multiplication of the bacillus in macrophages results in loss of tissue and impairment of nerve function (Bryceson and Pfaltzgraff, 1990., WHO, 1988., Jopling and McDougall, 1988).

### 1.2. History of the disease

There is a great deal of speculation about the history of man's relationship with leprosy. The oldest records which give an accurate description of the disease come from India, and may have been written as early as 600 BC (WHO, 1996). Chinese records have also been found describing what appears to be leprosy from a slightly later period (probably about 400 BC). The disease called leprosy in the Old Testament was probably not leprosy as we know it today, since the clinical description suggests a variety of other skin diseases and nerve damage is never mentioned.

The earliest concrete evidence of the presence of leprosy is seen in a Coptic mummy buried in the 5<sup>th</sup> century AD (Bryceson and Pfaltzgraff, 1990). It seems likely that

leprosy was widespread in northern and western Europe in the early Middle Ages reaching epidemic proportions in the 12<sup>th</sup> century and then slowly declined. Armaur Hansen was the first person to view leprosy bacilli under the microscope in 1873, and he concluded that the rod-shaped bodies he saw were the cause of leprosy (WHO, 1996; Bryceson and Pfaltzgraff, 1990).

### 1.3. Prevalence and distribution

Ten years ago the total number of leprosy patients varied between 10 and 15 million worldwide (Machin, 1988), but since the introduction of multi-drug therapy (MDT) in 1984 the number of registered cases and estimated total number of cases have now fallen below 1 million and 1.26 million respectively (WHO, 1996). However, there are no signs that the incidence is declining: about 500,000 new cases are detected annually (WHO, 1996) and the population at risk of contracting the disease remains very large (WHO, 1996). Approximately 2.4 billion people live in countries with a leprosy prevalence of more than 1 per 10,000 (WHO, 1995), and in about 16 countries leprosy is an important public health problem (WHO, 1996).

At present leprosy is most prevalent in tropical countries (WHO, 1988) although it was formerly very prevalent in cold countries (Bryceson & Pfaltzgraff, 1990). The number of multibacillary patients and the opportunities for exposure in a given area contribute to the spread of infection. It is well known that *M. leprae* doesn't cause disease in all humans exposed to it, nor does it produce the same degree of illness in those who do

become infected. A variety of risk factors like contacts with multibacillary patients, immune response of the host and living conditions have been presented to explain this variation. Improved living conditions help to prevent the spread of leprosy. For example, in spite of the absence of effective chemotherapy, the prevalence of leprosy declined sharply in Europe when living conditions improved after the Middle Ages (Bryceson and Pfaltzgraff, 1990).

#### 1.4. Leprosy in Ethiopia

Leprosy must be an old disease in Ethiopia (Prince, 1969), and is still a disease of major importance. High prevalence areas are mainly located in the central and north-central highlands, and low prevalence areas in the lowlands (Berhe *et. al.*; 1990). According to the Armauer Hansen Research Institute (AHRI) Annual Report (1993) the prevalence rate was reduced significantly after the introduction of MDT in 1984. After the introduction of MDT, a total of 71,291 patients were cured in Ethiopia (WHO, 1996). However, about 10,000 cases still require multi-drug treatment and more than 4000 new cases are detected annually. Efforts are being made to decrease the defaulter rate and to improve early case detection (WHO, 1996).

The problem of leprosy is far more serious than is indicated by the number of cases alone; the human suffering involved, resulting from physical deformities and related social problems, is intense. In Ethiopia about 4500 individuals have disabilities due to past or present leprosy (WHO, 1996). In addition, more than one third of all leprosy

patients face permanent and progressive physical and social disability (WHO, 1996). In Ethiopia, leprosy is still a stigma and leprosy patients are isolated from the community (Addise, 1992).

### 1.5. Biology of *M. leprae*

*M. leprae*, the first organism identified as the cause of a specific disease, was discovered by Armaur Hansen in Bergen Norway in 1873 (Bryceson & Pfaltzgraff, 1990). The organism is classified under the family *Mycobacteriaceae* and order *Actinomycetales* (Buchanan & Gibbon 1974). It is a straight or slightly curved rod-shaped, Gram positive, non spore forming, and strongly acid fast organism (Buchanan and Gibbons, 1974). Its acid fastness is believed to be due to the presence of mycolic acids in the thick cell wall of the bacterium. As far as is known, the bacterium reproduces naturally in man and in a few unusual species such as the nine banded armadillo (Yawalker, 1992). It has not yet been cultured *in vitro* (Hartskeerl *et al*, 1989). Thus knowledge concerning the basic biology of the bacterium including its metabolism and chemical structure is very limited (Colston, 1993). It will, however, multiply to a limited extent when inoculated in the mouse foot pad (Closs *et al*, 1979). The bacilli require cooler parts of the body for multiplication, hence the skin of the face, limbs, and the more superficial nerves are preferentially invaded (Hasting, 1985). Study in experimental animals (armadillo and mice) has shown that the optimum temperature for multiplication is 30°C (Rees, 1985). The multiplication time of *M. leprae* in the mouse foot pad is 12 to 14 days. This is uniquely slow even among the slow-growing Mycobacteria, such as *M. tuberculosis*,

which has a generation time of 20 hours (Yawalker, 1992).

#### 1.6. Mode of transmission

There is much misunderstanding about the mode of transmission of the disease. In China leprosy was believed to be divine punishment for having sexual intercourse with prostitutes (Jopling, 1971). In Ethiopia, many people believe that leprosy is not an infectious disease at all, but is inherited (Addisse, 1992). Although there are many factors involved in the transmission, the principal ones are a source of infection and contact with such a source. It is likely that the usual mode of transmission is via droplets or dust, and the upper respiratory tract is the normal portal of entry (Machin, 1988). In the past, lesions in the skin and nasal mucosa of leprosy patients have been recognized as sources of *M. leprae* (Meyers and Walsh, 1991). The present evidence indicates the much greater importance of the upper respiratory tracts of lepromatous leprosy patients (DeWit *et al*, 1993). It is very unusual to find leprosy bacilli on the surface of the skin, whereas a patient with active lepromatous leprosy will excrete about  $10^7$  -  $10^8$  bacilli in nasal secretions each day (Davy and Rees, 1974).

Epidemiological studies indicate that there is only human to human transmission of *M. leprae* (Machin, 1988). Large bacillary loads, usually 5,000 - 10,000 bacilli (Colston, 1993) are probably necessary to induce the disease in a target individual. Therefore, there is doubt that Arthropod vectors spread the disease, because they can carry only a small number of the bacilli (Machin, 1988). Most studies have shown that prolonged

contact with untreated multibacillary patients in the mid-borderline to lepromatous spectrum are of the highest epidemiological importance (Hasting, 1985). These patients have great number of bacilli in their nasal mucosa, and a great number of bacteria are expelled into the air when they sneeze, cough or talk (Yawalker, 1992; WHO, 1996). It is reasonable to accept that direct contact is perhaps far more effective in conveying the disease than indirect contacts, although the bacilli may be carried live on clothes, objects used by the patients, food, water, and dust (Desikan and Sreevatsa, 1995).

### 1.7. Spectrum of leprosy

Susceptibility to development of clinical leprosy may vary extensively from absolute refractoriness to a disseminated, progressive disease. About 5% of individuals who are infected get disease. Different types of responses to the infection are the result of the differences in the body's reaction to the bacilli (Bryceson & Pfaltzgraff, 1990). *M. leprae* causes disease by its ability to survive and multiply in macrophages (Mukherjee & Thomas, 1991). If macrophages are able to kill and destroy the bacilli soon after ingestion, there is little or no disease. Ridley and Jopling (1966) classified leprosy patients into a spectrum according to clinical and pathological evidence of immune responsiveness to *M. leprae*. In tuberculoid leprosy (TT), the patients mount a potent cell mediated immune response to *M. leprae* which includes macrophages activation, and are able to control the bacilli. However, in lepromatous leprosy (LL), cell mediated immunity is depressed. As a result, macrophages are incapable of destroying the bacilli, the host has no resistance at all, and acts as a perfect medium for the growth of *M.*

*leprae*. A few infected individuals (5%) exhibit selective unresponsiveness to antigens of *M. leprae* (Watson, 1989). In these patients the bacilli multiply in the skin and nasal mucosa, often to extraordinary numbers, (as many as  $10^{10}$ /gram of tissue). This can lead to paralysis and loss of tissue of hands and feet, paralysis of facial musculature, destruction of nasal structures, blindness, sensory loss, autonomic nerve damage, and testicular atrophy (Reiton, 1985).

Besides the two widely different types referred to above there is an intermediate form called borderline leprosy (BB). Borderline leprosy comprises a group of pathological features ranging from near tuberculoid, classified as borderline tuberculoid (BT) with a high resistance, to those symptoms more like that of lepromatous leprosy, classified as borderline lepromatous (BL).

Patients with borderline leprosy are relatively unstable unlike the polar forms of the diseases and may move either way along the spectrum. They have a tendency to develop acute reversal reactions (Lienhardt & Fine, 1994). This type of reaction is believed to be caused by a sudden increase in cell mediated immunity, so that antigens of *M. leprae* react with T-lymphocytes with a much greater intensity than before. This reaction is often observed in the early stages of anti-leprosy therapy (Becx-Blumnick *et al.*, 1992), and this can give rise to nerve destruction. Another type of reaction called erythema nodosum leprosum (ENL) occurs in polar and borderline lepromatous leprosy (Kifayet & Hussain, 1996). It is characterized by the formation of immune complexes

which are thought to cause demyelination and degeneration of the axon which ultimately leads to irreversible nerve damage (Kifayet & Hussain, 1996).

### 1.8. Detection of *M. leprae*

There is no quick and easy diagnostic method for the detection of leprosy. All the tools available today give false positive or false negative results. Most diagnosis are made by the recognition of skin or nerve lesions (WHO, 1995). Although early recognition is very important to prevent disability and deformities, it has been found that leprosy is one of the most difficult disease to diagnose in its early stages (Gupet, 1993., Baumgart *et al.*, 1993). Early diagnosis of leprosy in countries where leprosy is rare may be even more difficult, because the disease appears in so many different forms and a great deal of experience is needed for diagnosis (Yawalker, 1992., Bryceson & Pfaltzgraff, 1990).

#### 1.8.1. Clinical examination of leprosy

Diagnosis of leprosy is mainly based on clinical signs and symptoms. Information about the background of the patient may give some clues useful for diagnosis. In order to systematically evaluate for leprosy, information about the history of the person is particularly helpful. (Bryceson & Pfaltzgraff, 1990).

A good light is required for adequate physical examination to observe the evolution of skin lesions and any neurological abnormalities (Groenen *et al.*, 1995). However, the examiner may miss poorly visualized ill-defined patches in lepromatous leprosy and the

macule may be ambiguous, because loss of sensation may occur at a later stage (Baumgart *et al.*, 1993). In addition clinical examination may give false-negative results. According to Nagaraju (1994), under field conditions, 35% of leprosy cases were missed by field investigators in initial surveys using clinical examination.

### 1.8.2. Microscopic demonstration of acid fast bacilli

There is no quick and easy method for identification of *M. leprae*. In the past the tools available for the diagnosis of leprosy were very limited, and diagnosis had to be based mainly on clinical grounds. When the diagnosis is in doubt, it is generally confirmed by bacteriological examination, which involves microscopic observation of acid fast bacilli (AFB) in smears made from freshly cut skin surface (WHO, 1995). In addition, bacteriological examination may also be helpful in the classification of those cases of leprosy which cannot be easily classified on clinical signs alone (Groenen *et al.*, 1995).

Before implementation of multidrug therapy, classification was relatively less important because treatment with dapsone monotherapy was similar for all types of the disease, and only its duration varied (WHO, 1988). However, with the introduction of MDT for both paucibacillary (PB) and multibacillary (MB) leprosy patients, correct classification is much more important. Hence smear examination has now become critical for the choice of drug regimens and the success of chemotherapy (Groenen *et al.*, 1995).

Patients are classified as PB or MB according to the bacteriological index (BI). The bacteriological index indicates the density of bacilli in silt skin smears, and it includes both living and dead (fragmented) bacilli. The index ranges from 0 to +6, based on the number of bacilli that can be seen in an average field of vision (100X oil immersion). Each additional unit indicates a 10 fold increase in the number of AFB. For the purpose of control programs, TT, BT, and I (Indeterminate) patients, who usually have a bacillary index of 0 and are grouped as paucibacillary leprosy, while patients with a bacillary index of 1+ or more, as in the case of BB, BL, and LL, are grouped as MB patients (WHO, 1988).

For assessing the viability of *M. leprae*, the morphological index (MI) is widely used. It is defined as the proportion of solidly stained compared to the total number of bacilli, and is calculated after examining 100 or 200 bacilli lying singly. It provides a sensitive index of the viability of *M. leprae* in skin slit smears.

Although assessment of AFB is important for implementation of MDT, it is not sensitive and might not be suited for the diagnosis of carrier, PB, and some MB patients (Mcdougall, 1992). Moreover, demonstration of AFB doesn't indicate the presence of live bacilli especially in the case of treated patients. Significant proportion of MB patients show skin smear postivity after 2 years of MDT, but a follow-up study after eight years for relapse indicated the absence of live bacilli in 80% of treated patients (Jamet, 1995).

### 1.8.3. Cultivation of *M. leprae* in the mouse foot pad

Since leprosy is a chronic disease and the bacilli cannot be cultured *in vitro*, experimental infection of *M. leprae* in the mouse foot pad plays a crucial role in the assessment of live *M. leprae* after treatment (Yawalker, 1992). The reason for the susceptibility of these animals to leprosy may be the low temperature of their tissue coupled with depressed cell mediated immunity (Rees, 1985). The mouse foot pad system is used to determine viability (Shepard, 1985), to detect small number of *M. leprae*, and for drug sensitivity testing (Saito *et al.*, 1994). One of the problems of this system is that it cannot generate large quantities of *M. leprae* (Bryceson and Pfaltzgraff, 1990), since only limited replication takes place. However, it is ideal for the identification of new bactericidal chemotherapeutic agents and determination of their appropriate dosage and duration of treatment. For example, studies of ofloxacin-containing combined regimens in untreated LL patients showed that 400 mg daily ofloxacin was effective in rifampicin resistant cases (Rao *et al.*, 1994). In addition, the mouse foot pad system is used to determine the viability of the bacilli obtained from treated patients for the diagnosis of relapse. However, because the facilities for mouse inoculation are not accessible to most leprosy control programs, implementation of *in vivo* culture is not appropriate for the diagnosis of relapse under field conditions. This method is also important to determine whether the isolate in question is *M. leprae*. However, application of this techniques for early diagnosis, and large scale screening is impractical, because it is time consuming (it takes at least 10 months for the organisms to grow). Moreover, it is extremely expensive and is not quantitative (Rees, 1985).

#### 1.8.4. Lepromin test

The lepromin skin test has been widely used throughout the world for the last 70 years to assess the cell mediated immunity of patients and their contacts to *M. leprae* (Balckos and Lucas, 1995., Bryceson and Pfaltzgraff, 1990., Yawalker, 1992). The inoculum (Lepromin) is prepared from infected humans or armadillos infected experimentally with *M. leprae*. The inactivated inoculum (0.1 ml) is injected intradermally into the forearm. Two types of responses to lepromin take place. An early response, the Fernandez Reaction, which occurs in 24-48 hours is probably due to pre-existing delayed type hypersensitivity (Yawalker, 1992). The late Mitsuda reaction usually reaches its maximum size one month later, (Balckos and Lucas, 1995). The Mitsuda reaction may occur due to pre-existing immunity or cell mediated immunity (CMI) newly induced by the injected antigen.

The lepromin skin test is not specific for infected patients because most normal persons produce a positive Mitsuda reaction due to lepromin itself. According to Groenen *et al.* (1990) 87.6% of individuals without leprosy were lepromin positive. Skin testing is therefore of no diagnostic value but is useful in determining the position of patients on the immunological spectrum. Generally the lepromin test is positive in cases of TT, and BT patients but not in LL, BL and BB leprosy (Bryceson and Pfaltzgraff, 1990).

### 1.8.5. Antibody-based detection of *M. leprae*

There have been many studies attempting to identify and describe specific and sensitive diagnostic tools based on antigen-antibody reactions. Species specific antibodies to the capsular phenolic glycolipid (PGL-1) (Lal *et al.*, 1993), to the major 35 KD protein antigen (Parkash *et al.*, 1995), the 18 KD antigen (Vikerfors *et al.*, 1993), and to common Mycobacterial carbohydrate such as Lipoarabinomannan (LAM), have been studied widely.

Detection of species-specific antibodies in the serum of leprosy patients is not practical for diagnosis because antibody production is not uniform throughout the leprosy spectrum and is applicable only in lepromatous leprosy patients (Ilangumaran *et al.*, 1996). For example, when Parkash *et al.* (1995), used a serum antibody competition test for antibodies to the 35 KD protein molecule, 94.1% of MB patients were positive but the percentage decreased at the PB end of the spectrum. Hence, antibody-based detection can detect the majority of MB leprosy patients but a small number of PB leprosy patients.

Since untreated MB patients are seropositive, different investigators have examined the role of antibody levels in indicating responses to MDT. Changes in anti-PGL-1, anti-LAM, and anti-35 Kd antibody levels in response to MDT were measured by Roch *et al.* (1993) using on ELISA (enzyme linked immunosorbent assay). The rate of decline was less significant and the patients remained seropositive at the end of 2 years of

treatment. Furthermore, in some studies, healthy individuals from leprosy-endemic areas were found to be positive for PGL-1 antibodies (Lal *et al.*, 1993). Thus, serologically one cannot distinguish active leprosy and past infection (Sharma *et al.*, 1992). Even though *M. leprae* specific antibodies are not known to be protective their presence in the sera of leprosy patients has been evaluated for early detection (Abreu *et al.*, 1996), to monitor prognosis (Soares *et al.*, 1994), and as a marker of relapse (Sengupta, 1995), and reaction (Roche *et al.*, 1993).

#### 1.8.6. Antigen-based detection of *M. leprae*

The demonstration of *M. leprae* specific antigens in the serum and urine may indicate active infection (Mahon *et al.*, 1991), and may help in follow-up during chemotherapy (Sharma *et al.*, 1992). The PGL-1 antigen is found both in the serum (Young *et al.*, 1985), and in the urine (Mahon *et al.*, 1991). Species-specific PGL-1 antigen can be detected in concentrated urine by an antibody-based sandwich ELISA (Mahon *et al.*, 1991) in 92% of LL and 50% of BL, but only in 18% of BT patients. This result suggests that PGL-1 antigen detection provides a sensitive test for multibacillary leprosy, but the test is much less useful for the detection of paucibacillary cases.

Sharma *et al.* (1992), using the monoclonal antibody (ML 3oA2 IgG); tried to detect a non-specific cell wall antigen (MY3) of *M. leprae*. Fifty and 100% of 42 untreated PB and 48 MB leprosy patients were positive respectively. However, after six and 24 months of treatment, the antigen positivity became 50% for PB and 68% for MB patients.

Thus, this method is not applicable for detecting active infection and monitoring chemotherapy. In addition, it is less sensitive when it is compared to serological assays, especially at the paucibacillary end (Mahon *et al.*, 1991).

#### 1.8.7. (A) Molecular biology of *M. leprae*

The inability to grow *M. leprae* in the laboratory has greatly hampered studies on its biochemical and immunological characteristics as well as the mechanisms of pathogenicity (Clark-Curtiss *et al.*, 1985; Hasting, 1985). Recent advances in recombinant DNA technologies have allowed alternative approaches in the analysis and characterization of the *M. leprae* genome. *M. leprae* from experimentally-infected armadillo tissue was used for construction of gene libraries of *M. leprae* (Clark-Curtiss *et al.*, 1985) which have served as sources of *M. leprae* DNA. These libraries have been used for isolating and sequencing *M. leprae* genes which encoding major antigens using monoclonal antibodies (Young *et al.*, 1985), T cell clones (Villarreal-Ramos *et al.*, 1991), patient sera (Rinke de Wit *et al.*, 1993; Rinke de Wit *et al.*, 1992), and DNA probes (Abebe, 1993). Molecular biological studies of *M. leprae* have been used to investigate its relationships to other *Mycobacteria* (Dobner *et al.*, 1996), and to obtain large amounts of compounds such as protein antigens (Clark Curtiss *et al.*, 1985., and Eiglemeier *et al.*, 1993), or enzymes which can be used to study interaction with the host at the molecular level, to study the mechanisms of drug action (Sathish *et al.*, 1990), and for the development of diagnostic tools (Woods and Cole, 1990., Cox *et al.*, 1991).

The first *M. leprae* antigen that was identified by the recombinant DNA approach was the immunodominant 65 KD antigens (Thole, *et al.*, 1990). This antigen belongs to the family of GroEL heat shock proteins (Woods and Cole, 1989), but contain epitopes that are unique to various species of *Mycobacterium* (Plikaytis, 1992), and is useful for rapid identification at the species level (Telenti, 1993). In addition, proteins such as the 70, 65, 36, 35, 28, and 18 KD antigens have been well characterized using recombinant DNA technology. The objective of such antigen searches is to identify antigens that might be used for reliable diagnostic tests and might be important of vaccine development.

In the history of the molecular biology of *M. leprae*, one of the most striking advances has been the genome project conducted by the group of Stewart Code in Paris (Honore *et al.*, 1993). This project has been important for the availability of well characterized, ordered cosmid libraries of *M. leprae*, and for the study of the biological and physiological characteristics of *M. leprae*. This genome project have generated useful information about restriction maps, precise localization of genes and partial sequences (Eiglemeier, *et al.*, 1993).

Although *M. leprae* is considered a slow growing *mycobacterium*, its phylogenetic relationships among other *mycobacteria* based on the G+C contents and genome size was questionable. The genome of *M. leprae*, estimated to be  $2.2 \times 10^9$  daltons, is smaller than other *mycobacterium* species. In addition, most *mycobacteria* have between

60 - 67% G+C content in their DNA compared to 56% in *M. leprae*. DNA-DNA hybridization studies based on the 65 KD gene (McFadden *et al.*, 1990) indicate that the genome of *M. leprae* is only 20 - 30% identical to other *mycobacterial* species. This debate about the taxonomic position of *M. leprae* was settled by cloning and sequencing the 16S rRNA gene (Cox, *et al.*, 1991).

#### 1.8.7. (B) Nucleic acid-based detection of *M. leprae*

Molecular techniques have provided the potential for more sensitive and specific means of identifying pathogenic organisms (Saiki *et al.*, 1988), particularly where the causative agents are difficult to culture, or are slow growing, as is *M. leprae* (Colston, 1993).

Mycobacteria can be detected by the method known as DNA or RNA hybridization. Although this method is rapid it is not very useful due to its low sensitivity (McFadden, 1990),

Polymerase chain reaction (PCR) has undoubtedly been one of the most important advances in recombinant technology for a higher sensitivity of detection. PCR is a procedure for *in vitro* enzymatic amplification of a specific segment of DNA a billion fold. Using optimized PCR conditions of temperature, buffers, and purified DNA, these techniques are described to be sensitive enough to detect as few as 1 to 10 bacilli (Hartskreel, *et al.*, 1989).

Based on progress in understanding the gene sequences of *M. leprae*, several target sequences have been used for PCR. In general, these have either been DNA sequences which encode major antigens, non-antigen encoding sequences, or ribosomal RNA sequences (Cox *et al.*, 1991). Hartskreel *et al.* (1989), and De Wit *et al.* (1993) have described a PCR based assay in which a pair of primers amplify a portion of the *M. leprae* gene encoding the 36 KD proline-rich antigen from infected tissue. Polymerase chain reactions based on selective amplification of fragments of 36 KD, and, 18 KD, genes have shown a detection limit of 10 - 100 bacilli (Williams, *et al.*, 1990; Hartskreel, *et al.* 1989). Another potential PCR target is a specific sequence (RLEP) which is repeated with at least 28 copies (Woods and Cole, 1990) in the *M. leprae* genome. Yoon *et al.* (1993) detected *M. leprae* in about 61% of biopsies from leprosy patients negative for acid fast bacilli based on the amplification of the 372 base pair fragment of the repetitive sequence (RLEP) of *M. leprae*, indicating that PCR is more sensitive than this classical diagnostic methods for leprosy. The use of RLEP as a PCR target provides a theoretical advantage of higher sensitivity when it is compared with other targets (18 KD, 36 KD, and 65 KD groEL gene). Woods and Cole (1990), demonstrated the advantage of targeting the repetitive sequence over the groEL (65 KD) gene. When the DNA concentration of the PCR products for these samples were measured using scanning densitometry, the amount of the PCR product for the repetitive sequence was at least 15 fold higher than that of the PCR product of groEL gene. Even though, most reports of PCR from *M. leprae* DNA have shown a 100% specificity, not all paucibacillary patients yield a positive results, so the sensitivity is not 100%.

An alternative, potentially more sensitive technique, is PCR using RNA as the starting material. Cox *et al.* (1991) suggested that the 16S rRNA gene is an appealing target. The 16S rRNA gene contains some sequences that are unique to *M. leprae*. These differences can be exploited to detect *M. leprae* in infected individuals. In addition, several thousand copies of 16S rRNA are found per live bacterium, and their presence is likely to correlate better with the presence of live bacteria (Katoch, 1989). Thus detection of *M. leprae* in clinical specimens of the 16S rRNA by reverse transcription polymerase chain reaction (RT/PCR) theoretically may increase the sensitivity. RT/PCR is a modified technique for enzymatic amplification of RNA by the polymerase chain reaction. The basic principle is that complimentary DNA (cDNA) is synthesized from RNA using reverse-transcriptase, followed by enzymatic amplification by PCR. Kurabachew (1995) used specific 16S rRNA primers for detection of *M. leprae* and detected as few as 23 micro-organisms in test specimens. The sensitivity of this method in detecting *M. leprae* in skin biopsies was 93%; RT/PCR of skin biopsies from 14 leprosy patients gave positive results for 13 samples (Kurabachew, 1995).

The length and purity of amplified products can be determined by gel-electrophoresis. During application of current in the presence of an electrolyte solution, DNA molecules move uniformly towards the positive electrode due to their negative charge. Bands of DNA in the gel are stained with ethidium bromide and detected under ultraviolet light. However, the primers can be incorporated into nonspecific amplification products. These heterogenous products of various size are not easily seen on agarose gels. For specific

and sensitive detection, amplified DNA is denatured and transferred to nylon membranes, and detected using a labeled probe. Although these techniques are sensitive and specific, the detection of PCR products using gel-electrophoresis, Southern blotting, and probe hybridization is a complicated process. In addition, these assays are expensive, require equipment not generally available.

ELISA based detection can be adapted for detection of RT/PCR products, so as to combine the high throughput nature of microtiter plate with the sensitivity of PCR. In such post-PCR applications end-labeled primers are used for the specific capture and subsequent detection of amplified DNA.

#### **1.9. Objective of this project**

To develop a microtiter plate hybridization assay and detect *M. leprae* using 16S rRNA as an RT/PCR target.

## 2. Materials and Methods

### 2.1. RNA extraction

The extraction of RNA from *M. leprae*-containing armadillo tissue and biopsies from lepromatous leprosy patients was performed using RNA STAT-60™ (Tel Test "B", inc, Friends-wood, Texas). Fifty, 5  $\mu\text{m}$  slices were cut using a microtome (Laos 1720 cryostat, Wetzlar, Germany). Sterile petri dishes for the collection of thin sections and fresh microtome blades (type S35) for each slice were used to minimized sample contamination. Then 1 ml of RNA Stat 60™ was added. The slices were homogenized by pipetting up and down. After incubated at room temperature for 5 min the mixture was transferred to a clean 1.5 ml Eppendorf tube and vortexed vigorously for 20 seconds. The mixture was passed through a 21 gauge needle several times to insure homogenization and left at room temperature for an additional 5 min to allow the complete dissociation of nucleoprotein. Then 200  $\mu\text{l}$  of chloroform was added, the sample was shaken vigorously for 20 seconds and placed at room temperature for 3 min. The mixture were centrifuged at 12,000 rpm for 15 min at 4°C using a micro centrifuge (5415C Germany). After centrifugation, the colorless aqueous phase containing RNA was transferred to an Eppendorf tube which contained 500  $\mu\text{l}$  of isopropanol (Sigma). The tube was then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequent centrifugation at 7,500 rpm for 5 min at 4°C. The precipitate was

dried using a vacuum concentrator (Speed Vac concentrator SVC100H, Holland). Then the pellet was resuspended in 10  $\mu$ l diethylpyro carbonate (DEPC) treated RNase-free water (Sigma), and stored at  $-80^{\circ}\text{C}$ . The efficacy of the lytic buffer (RNA stat 60 <sup>TM</sup>) checked after DNAase and RNAase treatments (Kurabachew *et al.*, 1997).

## 2.2. cDNA synthesis

First strand cDNA synthesis was performed using AMV reverse transcriptase in a 20  $\mu$ l reaction mixture. The reaction mixture contained reverse transcriptase buffer at a concentration of 50 mM Tris-HCl, 8 mM  $\text{MgCl}_2$ , 30 mM KCl, pH 8.5 ( $20^{\circ}\text{C}$ ) (Boehringer mannheim, Germany), 6 mM dithiothreitol (DTT) (Stratagene), 1mM dNTP mix (0.25 mM dATP, 0.25 mM dGTP, 0.25 mM dTTP, and 0.25 mM dCTP) (Stratagene), 1 mM Oligo(dT)<sub>15</sub>, primer (Promega) 1mM anti-sense primer, 0.8 U of RNase inhibitor (Startagene) and 25 units AMV reverse transcriptase (Boehringer mannheim, Germany). The mixture was incubated at  $42^{\circ}\text{C}$  for 50 min. in the water bath. Enzyme was inactivated by heating to  $95^{\circ}\text{C}$  for 5 min followed by chilling on ice. Eighty  $\mu$ l of DEPC treated water was added, and the sample was stored at  $-20^{\circ}\text{C}$  until used in PCR reactions.

### 2.3. Primers and Probes

Primers and probes used in this study are listed on Table 1. All the primers and probes were obtained from School of Medicine Johns Hopkins University Baltimore MD.

P1 and P3 are sense and anti-sense primers that specifically amplify a 172 bp fragment of the cDNA for 16S rRNA of *M. leprae* (Kurabachew, 1995). P2 is also a sense primer which differ from P1 only with four bases. This primer doesn't overlap with the internal probe in the hybridization step in the Southern blotting and microtiter plate hybridization assay. Bio-P1 and Bio-P2 are 5' biotin labelled primers of P1 and P2 respectively.

Since mRNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is constituitivly expressed in human cells, GAPDH primers were used as internal control to detect inhibition of the reaction and for normalization of RNA among samples.

Three probes labelled with digoxigenin and FITC (Pr-1 labelled with digoxigenin, Pr-1 labelled with FITC, and Pr-2 labelled with FITC) in this study were used for indirect detection of *M. leprae* on the ELISA plate.

**Table 1. Primers and probes used in this study**

Primer	Type	Position	Sequence
GAPDH	S	367 - 384	5' ACC ACC ATG GAG AAG GCT GG 3'
GAPDH	A	875 - 894	5' CTC AGT GTA GCC CAG GAT GC 3'
16SrRNA(P1)	S	67 - 90	5' CGG AAA GGG TCT CTA AAA AAT CTT 3'
16SrRNA(Bio-P1)	S	67 - 90	5'BIO CGG AAA GGG TCT CTA AAA AAT CTT 3'
16SrRNA(P2)	S	64 - 86	5' CGA ACG GAA AGG TCT CTA AAA AA 3'
16SrRNA(Bio-P2)	S	64 - 86	5'BIO CGA ACG GAA AGG TCT CTA AAA AA 3'
16SrRNA(P3)	A	218 - 239	5' CAT CCT GCA CCG CAA AAA GCT T 3'
16SrRNA (Pr1)	Pr	85 - 112	5' CGC CAC TCG AGT ATC TCT AAA AAA GAT T 3'
16SrRNA (Pr2)	Pr	89 - 116	5' CGT TCG CCA CTC GAG TAA CTC TAA AAA A 3'
GAPDH Pr	Pr	571 - 600	5' GTG GAA GGA CTC ATG ACC ACA GTC CAT GCC 3'

**Key**

A = Anti-sense Primer

S = Sense Primer

Pr = Probe

1 TTGTTTGGAG AGTTTGATCC TGGCTCAGGA CGAACGCTGG CGGCCTGCTT

P1 & Bio-P1----->

P2 & Bio-P2----->

51 AACACATGCA AGTCGAACGG AAAGGTCTCT AAAAAATCTT TTTTAGAGAT

101 ACTCGAGTGG CGAACGGGTG AGTAACACGT GGGTAATCTG CCCTGCACTT

-----Probe-1

-----probe-2

151 CAGGGATAAG CTTGGGAAAC TGGGTCTAAT ACCGGATAGG ACTTCAAGGC

201 GCATGTCTTG TGGTGGAAAG CTTTTTGCGG TGCAGGATGG GCCCGCGGCC

←-----P3

251 TATCAGCTTG TTGGTGGGGT GACGGCCTAC CAAGGCGACG ACGGGTAGCC

301 GGCCTGAGAG GGTGTCCGGC CACACTGGGA CTGAGATACG GCCCAGACTC

351 CTACGGGAGG CAGCAGTGGG GAATATTGCA CAATGGGCGC AAGCCTGATG

401 CAGCGACGCC GCGTGGGGGA TGACGGCCTT CGGGTTGTAA ACCTCTTTCA

451 CCATCGACGA AGGTCTGGGT TTTCTCGGAT TGACGGTAGG TGGAGAAGAA

Fig.1. Partial Nucleic acid sequence of *M.leprae* 16S ribosomal RNA (Leisack *et al.*, 1990).

## 2.4. PCR amplification

PCR was performed in a total volume of 50  $\mu$ l using an automated thermal cycler (Hybaid, Omni Gene, UK). The reaction mixture (Boehringer Mannheim) contained 10 mM Tris-HCl (pH = 8.3); 15 mM MgCl<sub>2</sub>; 50 mM KCl; 0.25 mM each deoxy-nucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1 p mole each of the sense and anti-sense primer, 5  $\mu$ l of template cDNA, and 1 unit Taq DNA polymerase (Boehringer Mannheim) derived from *Thermophilous Aquaticus*. To prevent evaporation of liquid during thermal cycling, 40  $\mu$ l of mineral oil (Sigma) was layered on the top of the PCR solution. The amplification was performed for 40 cycles. Each cycle consisted of denaturation at 94°C for 2 minutes, annealing of primers at 60°C for 2 minutes, and primer extension at 72°C for 3 minutes. After the 40 cycles, the extension reaction was continued for another 10 minutes at 72°C to elongate product. The positive control consisted of *M. leprae* cDNA (0.21  $\mu$ g/ml) from a patient biopsy. Negative controls were reaction mixtures containing all reagents but no cDNA.

## 2.5. Detection of RT/PCR products

### 2.5.1. Gel electrophoresis

Agarose gel was prepared by adding 1.8g agarose in 100 ml tris borate EDTA buffer (TBE: 89 mM Tris base, 89 mM boric acid, and 2mM EDTA pH 7.4). Each well of the gel was loaded with 18  $\mu$ l of RT-PCR products and 2  $\mu$ l of 10X loading buffer (Sigma), and electrophoresed at the desired voltage (usually 50-250) according to the size of the gel. Gel were stained with ethidium bromide (0.5  $\mu$ g/ml) for about 20 minutes.

DNA bands were visualized with a medium wave UV transilluminator and photographed using a polaroid camera.

### **2.5.2. Southern hybridization and detection**

The DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH solution for 30 minutes and neutralized in 1 M Tris HCl, 1.5 M NaCl, pH 7.4 for 30 minutes. The DNA was transferred onto hybond N+ (Amersham) nylon membranes by capillary movements using 10X SSC (1X SSC = 0.219 M NaCl, 0.015 M sodium citrate, pH 7.0). The blot was then crosslinked using UV crosslinker for 35 second at 150 mJouls (Bio Rad, USA). Then FITC (Amersham Int.) labelled probe was diluted in hybridization buffer (Amersham Int.) at a concentration of 10 ng/ml and membranes were hybridized overnight at 47°C. This was followed by washing with 5X SSC, 0.1% (SDS) sodium dodecyl sulfate twice at room temperature for 5 min, and with 1X SSC, 0.1% SDS at 47°C for 15 min with constant agitation and without agitation for a further 15 min twice. The membrane was then incubated with blocking reagent (supplied) at a 1:20 dilution in 0.15 M NaCl, 0.1 M Tris base, pH 7.5 for 1 hour. This was followed by washing with 0.4 M NaCl, 0.1 M Tris base, pH 7.5 for one minute and incubation with antibody conjugated horseradish peroxidase at a 1:1000 dilution for one hour at room temperature. After washing with 0.4M NaCl, 0.1 M Tris base, pH 7.5, equal amounts of detection solution 1 and detection solution 2 (Amersham Int.) were added directly to the blot and incubated for 1 min at room temperature. Then the blot was covered in plastic wrap and exposed to autoradiography film (Hyper film-ECL, Amersham Int.) for

5 - 30 min. The film was then processed in Kodak GBX developer and fixer (Kodak, USA).

### **2.5.3. Probe labelling for ECL**

The probe was labeled at 3' end following the protocol of the ECL<sup>TM</sup> 3'- oligolabelling and detection system (Amersham Int.). One hundred  $\mu$ mole oligonucleotide, 5  $\mu$ l fluorescein-11-dUTP, 8  $\mu$ l cacodylate buffer, 56  $\mu$ l water (supplied), and 8  $\mu$ l of terminal transferase were mixed and the reaction mixture was incubated at 37°C for 1 hour. Then labelled probe was placed on ice for immediate use or stored at -20°C for later use.

## **2.6. ELISA-based detection of RT/PCR products**

One direct and two different types of indirect colorimetric microtiter plate hybridization assays were tested. For the direct microtiter assay, 11 different types of blocking reagents were tested. Four different streptavidin coating buffers were tested for indirect microtiter plate assays.

### **2.6.1. Direct microtiter plate assay**

#### **A Direct colorimetric microtiter plate assay**

Initially microtiter plates (Dynatech Microtiter<sup>R</sup>, USA) were coated with 100  $\mu$ l of biotinylated RT/PCR product (40  $\mu$ l RT/PCR + 60 $\mu$ l water) and incubated overnight at 4°C. To remove unbound RT/PCR products the plate was washed at least twice with washing buffer [PBS (137 mM NaCl, 2.7 mM KCl, 9.3 mM Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 1.4 mM

$\text{KH}_2\text{PO}_4$  pH 7.5) with 0.05% vol/vol Tween-20] at room temperature. To block non-specific binding, 200  $\mu\text{l}$  of blocking reagent (BSA/DNA/Tween-20) was then added to the wells and incubated overnight at 4°C. After washing the plate twice, 100  $\mu\text{l}$  avidin-conjugated alkaline phosphatase (1:1000 in blocking buffer), was added to each well, and plates were incubated for 4 hours at room temperature. Following washing of wells twice, p nitro phenol phosphate (pNPP) tablets (Sigma) were diluted in 20 ml of distilled water and 100  $\mu\text{l}$  was added in each well, and incubation was continued for 2 hours in the dark. After two hours, the reaction was stopped with 100  $\mu\text{l}$  of 3N NaOH. The optical density (OD) of the resulting yellow color was measured at 405 nm. The values were expressed as net ODs after the OD of the buffer blank was subtracted.

#### **B Selection of blocking reagent**

Eleven different types of blocking reagents were tested (Table 2). To select the best blocking reagent the microtiter plate wells were incubated with 200  $\mu\text{l}$  of blocking reagents overnight at 4°C followed by washing with PBS with 0.05% vol/vol Tween-20.

This was followed by addition of 100  $\mu\text{l}$  of avidin conjugated with alkaline phosphatase and incubation for one hour at room temperature. Then 100  $\mu\text{l}$  pNPP/well was added and plates were incubated for 2 hours before the optical density was measured at 405 nm using an ELISA reader (Bio-Rad Mississauga, Ontario Canada).

**Table 2. Blocking reagents used in this study.**

Block	Buffer	Concentration
Milk	PBS	5% Milk (wt/vol) with 0.02% sodium azide
Tween-20	PBS	0.2% Tween with 0.02% sodium azide
FCS	PBS	10% FCS with 0.02% sodium azide
BSA	PBS	2.5 mg/ml BSA with 0.02% sodium azide
DNA	PBS	100 µg/ml with 0.02% sodium azide
BSA/DNA	PBS	2.5 mg/ml BSA/100 µg/ml DNA with 0.02% sodium azide
Tween-20/FCS	PBS	10% FCS/0.2% Tween-20 with 0.02% sodium azide
BSA/DNA/Tween-20	PBS	2.5 mg/ml of BSA/ 100 µg/ml of DNA/ 0.2% Tween with 0.02% sodium azide
FCS/Tween-20/DNA	PBS	2.5 mg/ml of BSA/ 10% FCS/ 100 µg/ml of DNA with 0.02% sodium azide
FCS/BSA/Tween-20	PBS	10%FCS/2.5 mg/ml of BSA/0.2% Tween with sodium azide
FCS/BSA/DNA	PBS	10%FCS/2.5 mg/ml of BSA/100 µg/ml DNA with 0.02% sodium azide

## 2.6.2. Indirect assay with blocking reagent

### A Indirect assay with blocking reagent

This assay was adapted from McDonnell *et al.*, (1995). Microtiter plates were coated with 50  $\mu$ l streptavidin (Sigma Chemical, St. Louis, Mo, USA ) at 100  $\mu$ g/ml in 0.1 N carbonate/bicarbonate buffer (Sigma, pH 9.6 at 25<sup>o</sup>C) overnight at 4<sup>o</sup>C. After two washes with water and one with 250  $\mu$ l PBS/Tween-20, blocking buffer (PBS/Tween-20 with 100  $\mu$ g DNA & BSA at 0.1% w/v) was added for 1 hour at room temperature. This was followed by two more washes with PBS/Tween-20. Then 20  $\mu$ l of varying dilutions of biotinylated RT/PCR product were mixed with 30  $\mu$ l 5x SSPE (1X SSPE = 0.22 M NaCl, 0.01 M NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 0.00126 M EDTA pH 7.4), added to the microtiter plate, and incubated for 1 hour at room temperature. This was followed by two more washes in PBS/Tween-20 and denaturation using 1N NaOH for 3 min at room temperature. After three washes with PBS/Tween-20, 50  $\mu$ l of internal probe labelled with digoxigenin (5  $\mu$ g/ml) in PBS/1% BSA was added and plates were incubated for 1 hour at 37<sup>o</sup>C. Next, 50  $\mu$ l of anti-digoxigenen-antibody-alkaline phosphatase conjugate, Fab fragments (Boehringer Mannheim, Germany) at a dilution of 1:500 were added for 45 min at 37<sup>o</sup>C, and this was followed by three washes with PBS/Tween-20. Then 50  $\mu$ l substrate (pNPP) (Sigma) at 1 mg/ml were added. The OD after two hours or overnight incubation was quantitated at 405 nm in a model 3350 plate reader (Bio-Rad, Mississauga, Ontario, Canada).

## **B Selection of coating buffer**

Four different types of streptavidin coating buffer (PBS, carbonate/bicarbonate, 6X SSPE (sodium chloride with sodium phosphate and EDTA), and 6X SSC (sodium chloride with sodium citrate)) were tested initially. In subsequent experiments, the coating buffer and blocking reagent which gave the strongest signal with low background were used.

## **C Probe labelling with digoxigenin**

The probe was labelled with digoxigenin at the 3' end according to the manufacturer's protocol (Boehringer Mannheim). Initially 4  $\mu$ l of reaction buffer containing, 5mM  $\text{CoCl}_2$  solution, 100 p-mole oligonucleotide corresponding to 100 p-mole unlabelled control oligonucleotide, 5 p-mole DIG-dUTP, 50 p-mole dATP solution, and 50 units terminal transferase mixed together and the volume was adjusted to 20  $\mu$ l with sterile redistilled water. Then the mixture were incubated at 37°C for 15 minutes and placed on ice, 1  $\mu$ l of glycogen and 200  $\mu$ l of 0.2 M EDTA were added. The oligonucleotide was precipitated with 4 M LiCl and 75  $\mu$ l ethanol at -70°C overnight. After centrifugation at 12,000g rpm, the pellet was washed with 50  $\mu$ l 70% (v/v) cold ethanol, dried under vacuum, and dissolved in 20  $\mu$ l of redistilled water and stored at -20°C until used.

### 2.6.3. Indirect assay without blocking reagent

#### A Indirect assay without blocking reagent

Microtiter plates were coated with 50  $\mu$ l of Streptavidin (100  $\mu$ g/ml), covered with Titertek plate sealers (INC Biomedical Inc, Costa Mesa) and incubated overnight at 4°C. Plates were washed three times with PBS (200 mM NaCl, 2.7 mM KCl, 10.43 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) with 0.5% Tween-20 (Sigma) using a micro-plate washer (Titertek S8/12, U.K). Then 5  $\mu$ l of biotin-labelled PCR product was mixed with 20  $\mu$ l of hybridization buffer (6x SSPE containing 1.3 M NaCl, 60 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 7.6 mM EDTA, pH 7.4) 5X Denhardt's solution, and 0.02% sodium dodecyl sulfate (SDS) pH 7.2, and incubated at room temperature for 30 minutes. This was followed by washing with PBS/Tween-20 5 times at room temperature. Then wells were incubated with 100  $\mu$ l of 1N NaOH at room temperature for three minutes and washed 5 times with PBS/Tween-20 at room temperature.

Fluorescein (FITC)-labelled probe was diluted in hybridization buffer (50 ng/ml) and 50  $\mu$ l was added to the microtiter plates. This was followed by incubation for 30 minutes at room temperature and then washing with PBS/Tween-20 three times at room temperature. Plates were then washed under stringent conditions using 0.01X SSPE with 0.1% SDS at room temperature for five minutes, followed by 5 washes with PBS/Tween-20 at room temperature.

Anti-fluorescein antibody Fab fragments, conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) was diluted 1:500 in PBS/0.1% BSA (Sigma fraction V). Then 50  $\mu$ l was added to microtiter wells followed by incubation at room temperature for one hour and 5 washes with PBS/Tween-20 at room temperature. Fifty  $\mu$ l of the substrate (pNPP, 1 mg/ml, Sigma) was added to each well, incubated in the dark for 2 hours or overnight, and OD was measured at 405 nm.

**B Selection of probe and further optimization of incubation temperature, time, and washing buffer for indirect ELISA without blocking**

Three different types of probes, one labelled with digoxigenin (Pr1-dig), and two with FITC (Pr1-FITC and pr2-FITC) were tested. In addition, we examined the shortest time needed for maximum signal using different temperatures. Different concentration of streptavidin (0.1, 1, 10, and 100 ng/ml) in carbonate/bicarbonate buffer were tested. Different temperatures (room temperature, 37°C, and 42°C) and different incubation times (10 min, 30 min, and one hour) were tested. In other experiments, double stranded RT/PCR was denatured for 1, 2, 3, 4, or 5 min with 1N NaOH. In addition, during the hybridization, different concentration of probe (25, 50, 100, and 200 ng) were hybridized at room temperature, 30°C, or 42°C. To select the optimum concentration of salt in the post hybridization washes varying concentration (0.001X SSPE, 0.005X SSPE, 0.01X SSPE, 0.05X SSPE, 0.1X SSPE, 0.5X SSPE, and 1X SSPE) different temperatures (Room temperature (RT), 37°C, and 42°C) were tested.

## 2.7. Sensitivity of ELISA-based detection of RT/PCR products

### 2.7.1. Separation and counting of *M. leprae* from mouse foot pad and armadillo liver

The method for quantitative recovery of *M. leprae* cells from infected Armadillo liver and Mouse foot pad was adapted from Clark-Curtiss *et al.* (1985). Four grams of infected mouse foot pad was placed into a sterile hand homogenizer with 4 ml of distilled water and 1:100 dilution of 10 mM Phenyl methyl sulfonyl fluoride (Sigma). The tissue was homogenized, then passed through a sterilized wire mesh tea strainer (diameter 3 inches) to remove tissue debris, and slowly layered onto a Percoll (Sigma) gradient (40-100%) for density separation of *M. leprae* cells from mouse foot pad tissue residue. After centrifugation for 6 hours at 12,000 rpm (Sorvall Superspeed RC2-B, Swing rotor type HB-4), the *M. leprae*-containing band was carefully removed from each tube with a pasture pipette, washed with distilled water, and centrifuged three times at 1,500 rpm for 10 min (IEC Centra-3RS, England) to removed the Percoll. *M. leprae* were counted after Ziehel-Neelsen staining.

### 2.7.2. Sensitivity testing

To check the sensitivity of the indirect assay without blocking reagents we used two different approaches. First, amplified RT/PCR products were serially diluted in water and then subjected to the ELISA-based detection. Alternatively, known numbers of *M. leprae* purified from mouse foot pad were diluted and subjected for RNA extraction, cDNA synthesis and PCR amplification. Then the OD was measured using an ELISA

reader.

### 2.8. Detection of RNA in tissue specimens using GAPDH primers

RNA extraction and cDNA synthesis was performed for each sample following the standard procedure. Then 10  $\mu$ l of cDNA was subjected to PCR amplification using Glyceraldehyde Phosphate Dehydrogenase (GAPDH) primers. Samples were heated to 94°C for 3 min initially, then 94°C for 1 min., 60°C for 2 min., and 72°C for 1 min for 35 cycles. Extension was continued for 5 min. at 72°C. After amplification, 20  $\mu$ l of each sample was electrophoresed through an agarose gel and visualized after ethidium bromide staining.

### 2.9. Detection of *M. leprae* in patient biopsies

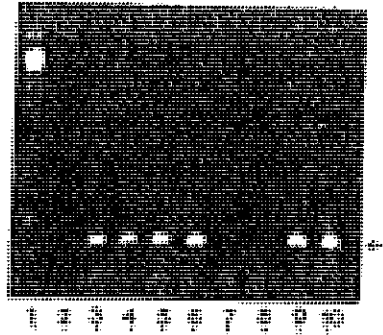
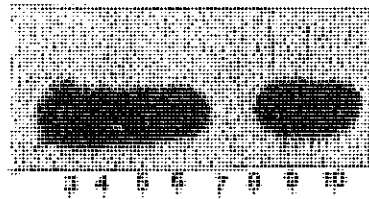
Biopsy samples were obtained from skin lesions of non-immunized leprosy patients classified as MB or PB based on clinical diagnosis of slit skin BI according to the Ridley and Jopling scale. The study group included 36 multibacillary and 25 paucibacillary patients.

The cutoff for positive values was defined as  $\pm 3$  standard deviations above the mean of the control values.

### 3. Results

#### 3.1. Selection of primers

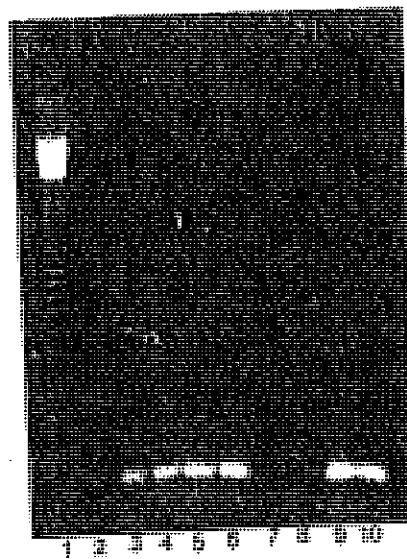
To optimize the assay, RNA was extracted from an infected armadillo liver sample and from known lepromatous leprosy patient biopsies. Yields of RNA were 1.885  $\mu\text{g}/\text{ml}$  and 6.155  $\mu\text{g}/\text{ml}$  respectively. The RNA also considered pure, since the ratio of nucleic acid to protein at OD<sub>260</sub> and OD<sub>280</sub> was 1.8. After synthesizing cDNA from the RNA two PCR reaction mixes containing equal amounts of cDNA with different primers were set up. The first mix contained specific sense (P1) and anti-sense (P3) primers for 16 S rRNA of *M. leprae* described by Kurabachew (1995), and the second mix contained another sense primer (P2) and (P3). Agarose gel electrophoresis of PCR products are shown on Fig 2A. Successful amplification was seen in all samples. The positive control and the negative control also gave the expected results. A Southern blot analysis (Fig. 2B.) confirmed the specificity of the PCR.

**A****B**

**Fig. 2. Comparison of P1 and P2 sense primers. (A)** Agarose gel electrophoresis of RT/PCR products after 40 cycles. The arrow indicates the 172 bp and 176 bp 16S rRNA amplification products. Lanes 1-molecular weight markers of the amplified products, 3-cDNA from armadillo liver amplified with P1 + P3, 4-cDNA from lepromatous patient amplified with P1 + P3, 5-cDNA from armadillo liver amplified with P2 + P3, 6-cDNA from patient biopsy amplified with P2 primer + P3, 7-negative control with P1 + P3, 8-negative control with P2 + P3, 9-positive control with P1 + P3, and 10-positive control with P2 + P3 **(B)** Southern blot of the above products.

### 3.2. PCR efficacy of biotinylated and non-biotinylated primers

To determine whether labelling the primer with biotin inhibited the amplification reaction, we added equal amounts of cDNA to PCR mixes containing either non-biotinylated or biotinylated primer. As shown in Fig. 3. on visual inspection, biotinylated primers (5 and 6) actually gave a stronger signal than the non-biotinylated primer (3 and 4).



**Fig. 3. Comparison of PCR product obtained using biotinylated and non-biotinylated primer.** Lane 1 - molecular weight markers, 3&4 - amplified products using non-biotinylated primers from armadillo and lepromatous leprosy patient, 5&6 - amplified products using biotinylated primers from armadillo and lepromatous leprosy patient, 7&8 - negative control for non-biotinylated and biotinylated primers respectively, 9&10 - positive control for non-biotinylated and biotinylated primers.

### 3.3. Selection of blocking reagents for direct assay

In order to optimize the conditions for detection of RT/PCR products a total of eleven different types of blocking reagents were tested. As shown in Table 3, BSA/DNA/Tween-20 gave the lowest background for the negative control. Since a low background was seen consistently using this blocking reagent, it was selected for further investigation in the direct and indirect microtiter assay.

**Table 3. Comparison of background levels using different blocking reagents in the direct assay.**

Block	OD
Milk	2.01
Tween	0.54
FCS	0.66
BSA	0.46
DNA	0.64
BSA/DNA	0.45
FCS/Tween	0.11
BSA/DNA/Tween	0.01
FCS/DNA/Tween	0.17
FCS/BSA/DNA	0.15
PBS	0.64

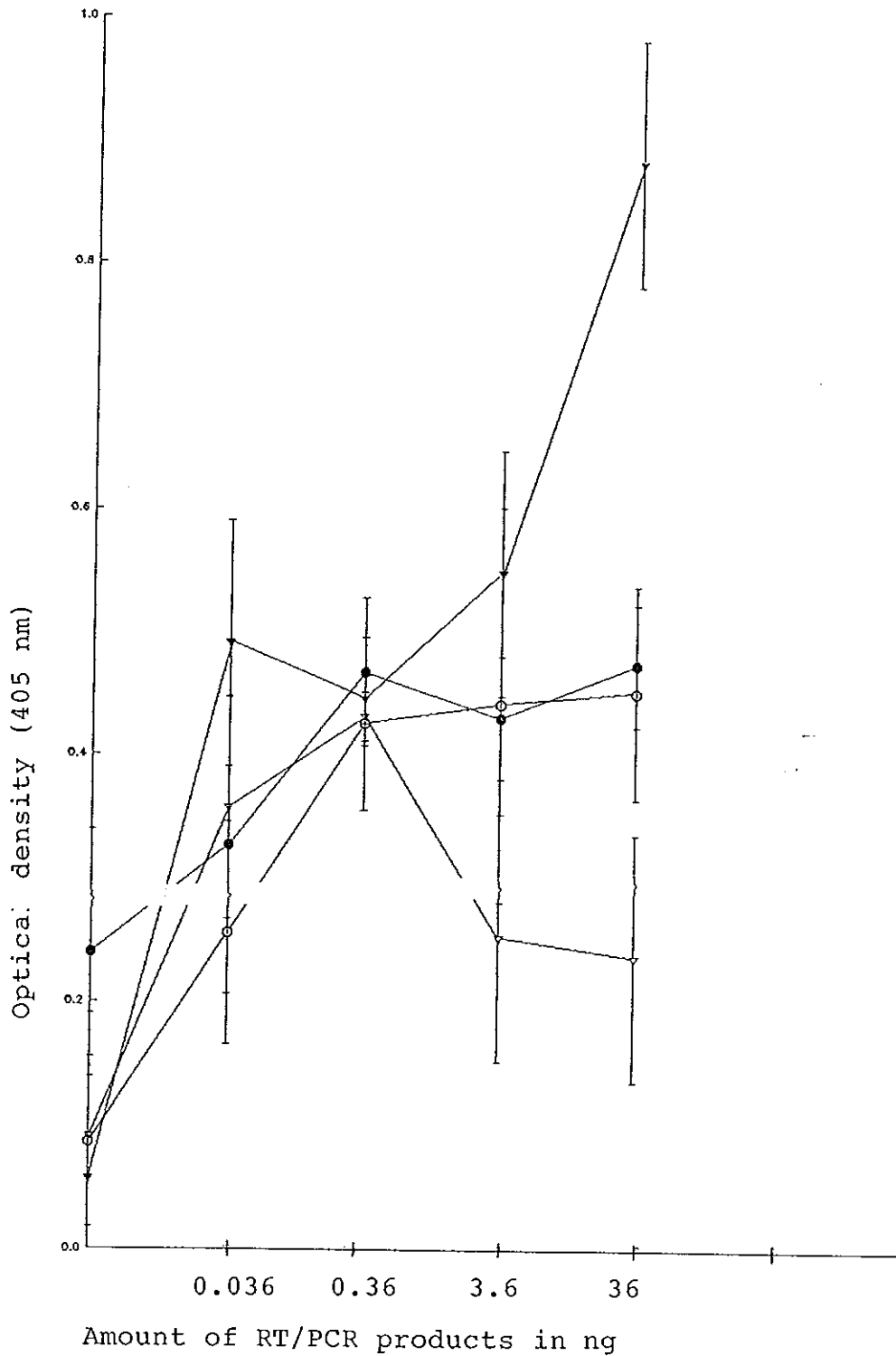
Key

OD = Optical Density

Block = Blocking reagents

### 3.4. Selection of coating buffer

Different types of coating buffers were tested using indirect assay with blocking reagents to choose the buffer that gave not only a lowest background but also a consistently strong signal. A total of four different types of coating buffer (PBS, carbonate/bicarbonate buffer, 6X SSPE, and 6X SSC) were examined. BSA/DNA/Tween-20 was used as a blocking reagent. All the buffers gave low background at lower concentrations except SSC. As shown in Fig. 4 when the DNA concentration increases, the optical density of SSPE fluctuates. PBS and carbonate/bicarbonate gave lower backgrounds with strong signals at the lower concentrations of RT/PCR product. In contrast, when the concentration of the RT/PCR products increased, carbonate/bicarbonate buffer gave the best result.



**Fig. 4. Comparison of streptavidin coating buffers.** Ten fold dilutions of RT/PCR products were prepared from 36 ng of the stock using different coating buffer (PBS (○), carbonate/bicarbonate (▼), SSC (●), and SSPE (▽) for the microtiter plate hybridization assay at varying concentrations of amplified product.

### 3.5. Comparison of different types of ELISA-based detection for RT/PCR products

Equal concentration of ten fold diluted RT/PCR products from armadillo derived *M. leprae* were subjected to detection using the three systems. The major difference among the three systems were the probes, the presence or absence of blocking reagents, and the incubation period. Significant differences were observed between the direct and indirect assays (Fig. 5). In the case of the indirect microtiter assay, the background was almost zero and the optical density increased with the amount of RT/PCR products added. However, the color change in the direct microtiter assay did not steadily increase with increasing concentration of RT/PCR products. In addition, when we compared the indirect microtiter plate assay with blocking reagent with the indirect microtiter plate hybridization assay without blocking reagents both systems gave a strong signal at higher concentrations of RT/PCR products. However, when the concentration was decreased the later become more and more sensitive. Hence, indirect microtiter plate assay without blocking reagent was selected for the subsequent optimization process.

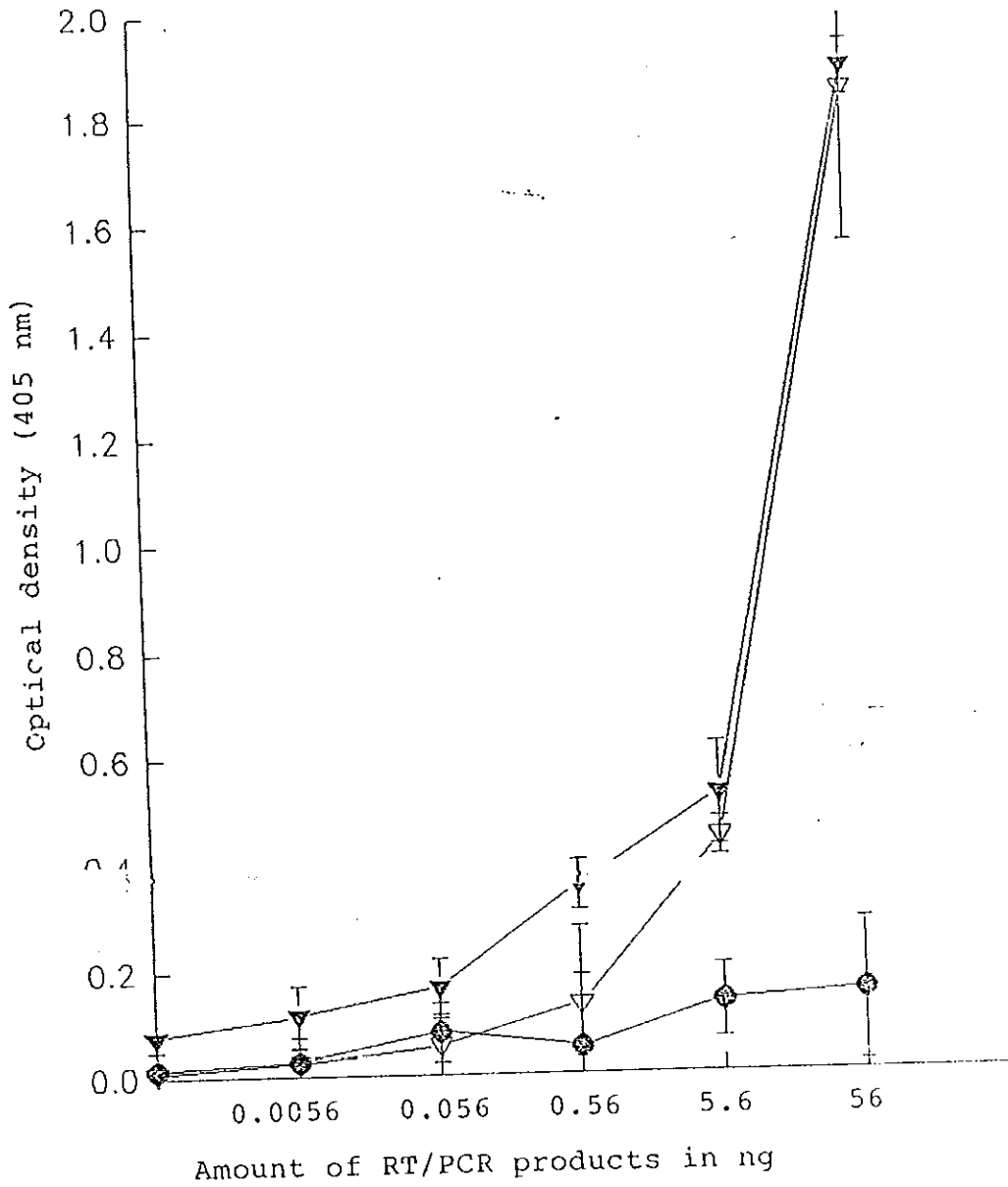
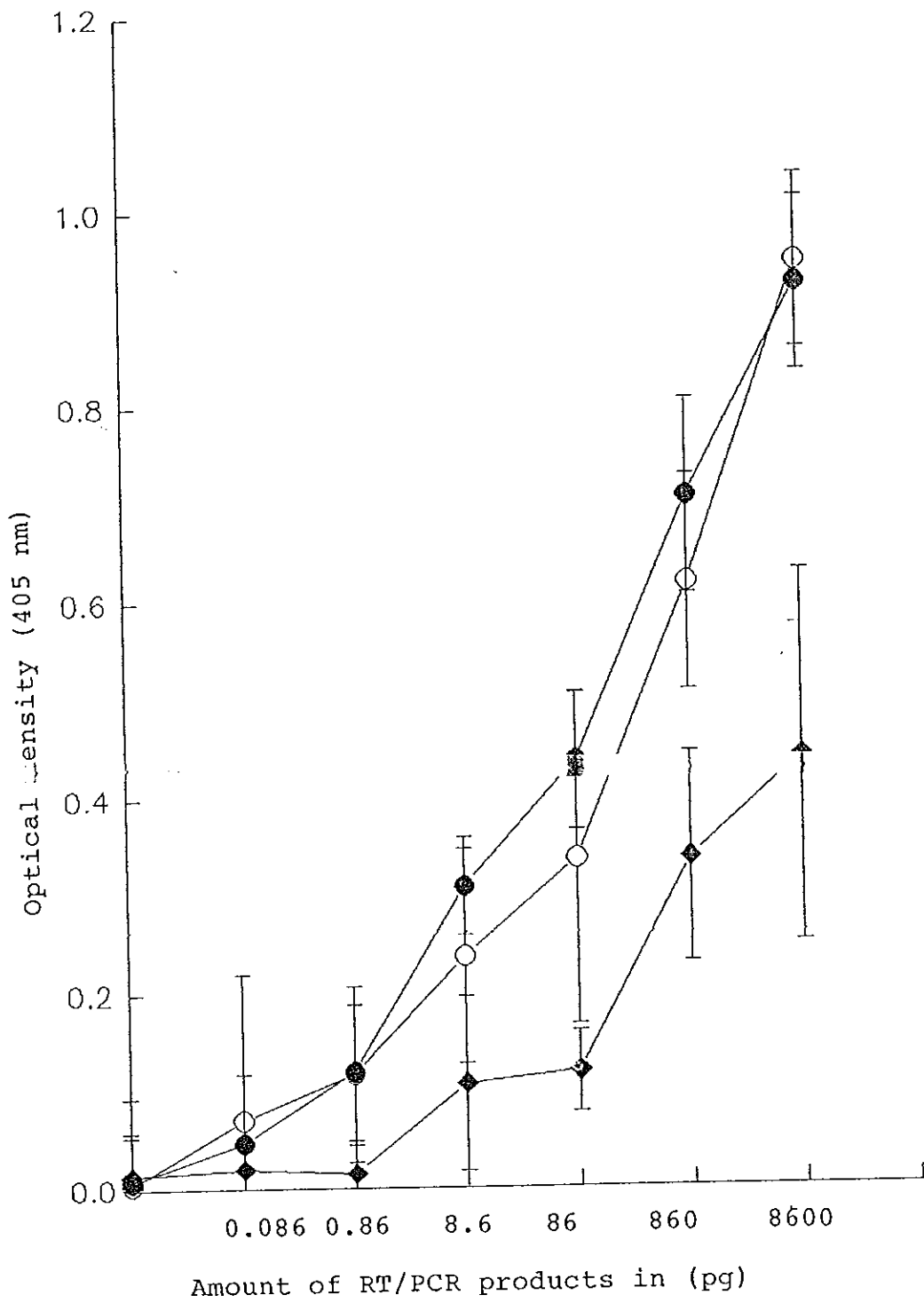


Fig. 5. Comparison of different microtiter assays. RT/PCR products amplified with P2 and P3 primers were subjected to detection by the direct microtiter plate assay (●), the indirect colorimetric assay with blocking reagents (∇), and the indirect colorimetric assay without blocking reagent (▽) using a digoxigenin labelled probe. Ten fold dilutions were prepared from 56 ng of RT/PCR product.

### 3.6. Selection of probes

After the selection of the indirect microtiter plate hybridization assay without blocking reagents for subsequent experiments, we tested three different probes labelled with two different reagents. These included one digoxigenin labelled probe (Pr1-dig) and two FITC labelled probes [Pr1-FITC (FITC-1) and Pr2-FITC (FITC-2)]. Equal concentrations of serially diluted RT/PCR products were applied to the plates. FITC-1 and FITC-2 gave strong signals and low background (Fig. 6.). We selected FITC-2 for our subsequent experiments since this probe didn't contain nucleotides which overlapped with the 5' primer.



**Fig. 6. Probe selection.** RT/PCR products were amplified using P4 + P5 primers and subjected to detection using the indirect microtiter plate hybridization assay without blocking reagent and digoxigenin labelled probe (◆), FITC-1 (O), and FITC-2 (●). Ten fold dilutions of RT/PCR products were used from 86 ng stock.

### 3.7. Further optimization of temperature, washing buffer, and incubation time

To select the optimum concentration of streptavidin different concentration of streptavidin (0.1, 1, 10, and 100 ng/ml in 50  $\mu$ l carbonate/ bicarbonate buffer) were added to wells of the microtiter plate and incubated overnight at 4<sup>o</sup>C. Then equal amount of amplified products were added to wells and the detection was completed. At lower concentration of RT/PCR product, the background for all streptavidin concentrations were similar, (0.04, 0.05, 0.03,0.04, and 0.04 for 0.1, 1, 10, and 100 ng streptavidin respectively), but when the concentration of the RT/PCR product increased, 100 ng streptavidin gave the strongest signal. Hence we used this concentration throughout our experiments.

Since the signal for the biotin-labelled PCR products was not affected by increasing temperature and incubation time, we selected room temperature for incubation.

Two concentration of probe (50 and 100  $\mu$ g/ml) gave stronger signals in comparison with the others. Eventually wash conditions were examined at different temperatures (42<sup>o</sup>C, 37<sup>o</sup>C, RT) and decreasing concentrations of SSPE (0.001X, 0.005X, 0.01X, 0.05X, 0.1X, 0.5X, 1X) with known positive and negative RT/PCR samples. The best results was observed with 0.01X and 0.05X SSPE at room temperature.

### 3.8. Assessment of sensitivity

#### 3.8.1. Assessment of sensitivity using serially diluted RT/PCR products

A known concentration of an RT/PCR to 16S rRNA products was serially diluted and subjected to colorimetric detection. As shown from the Fig. 7, the ELISA-based assay detected as little as 5.6 pg of RT/PCR product.

#### 3.8.2. Assessment of sensitivity using serially diluted bacilli

*M. leprae* were purified from mouse foot pad and the number of bacilli estimated by microscopic counting after Ziehl-Neelsen staining. A known number of *M. leprae* ( $10^6$  -  $10^5$ ) was serially diluted and then subjected for RNA extraction, cDNA synthesis, and PCR. Using the ELISA-based detection, as few as 10 bacilli were detected (Fig. 8.).

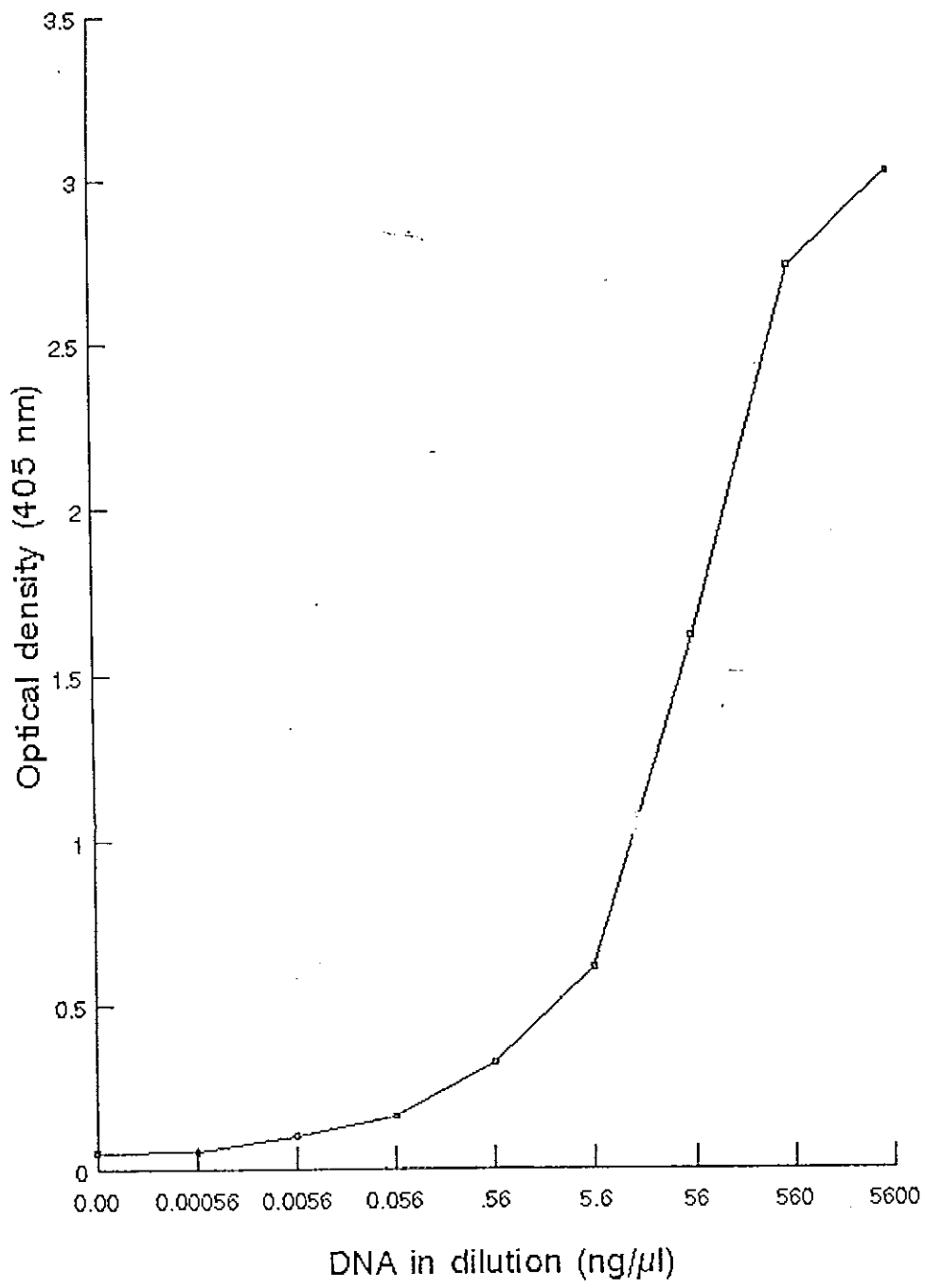
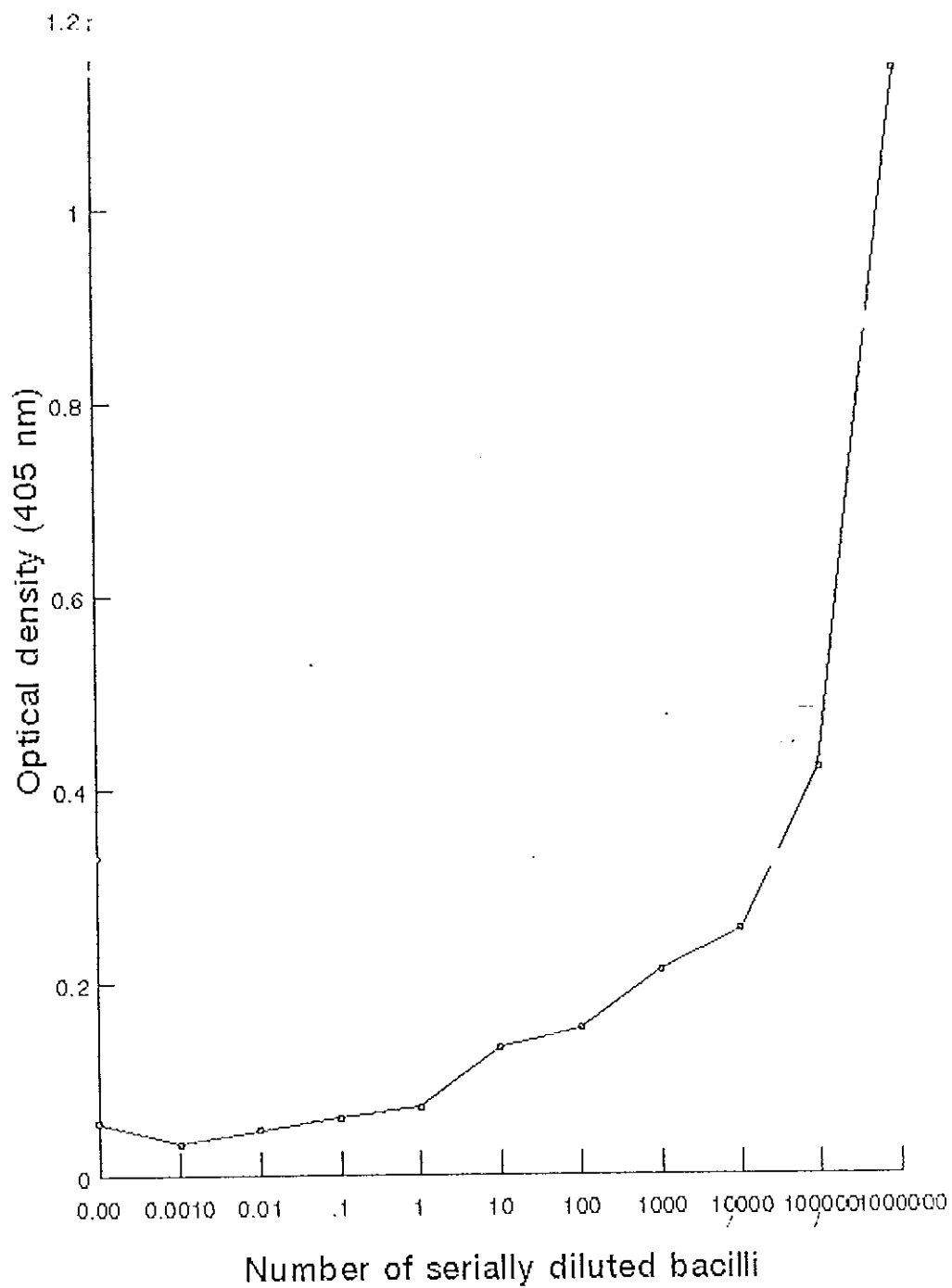


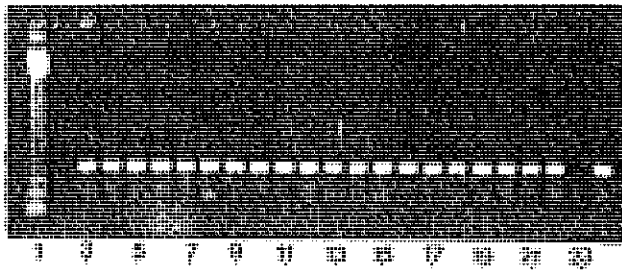
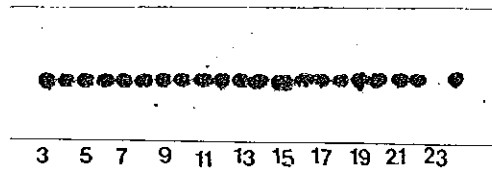
Fig. 7. Sensitivity of the colorimetric microtiter plate hybridization assay tested with serially diluted RT/PCR product.



**Fig. 8. Sensitivity of colorimetric assay in detecting serially diluted bacilli.** Bacilli were subjected to RNA extraction, cDNA synthesis, and PCR amplification using Bio-P2 + P3 primers. Products were run in the indirect microtiter plate assay without blocking reagent.

### 3.9. Assessment of *M. leprae* in the control skin biopsies

The specificity of the ELISA-based detection was demonstrated using skin biopsies obtained from 16 non-leprosy patients (patients with skin diseases other than leprosy) and 4 biopsies from normal individuals. First the specimens were subjected to RT/PCR amplification of a known internal control (GAPDH) using GAPDH primers. Amplification products were electrophoresed through 1.5% agarose gels (Fig. 9A.) and subjected to Southern blotting (Fig. 9B.). Then the same sample specimens were subjected to PCR amplification of the 16S rRNA using biotin labelled S2 primer (Bio-P2) and anti-sense primer (P3) and analyzed after electrophoresis (Fig. 10A.), Southern blotting (Fig. 10B.) and by the ELISA-based detection system (Fig. 11.).

**A****B**

**Fig. 9. RT/PCR amplification of GAPDH fragment from skin biopsies of non-leprosy patients and healthy individuals. (A) Ethidium bromide stained agarose gels. Lanes 1 - molecular weight markers, 3 to 18 - non-leprosy patients, 19 to 22 - healthy individuals, 23 - negative control, 24 - positive control. (B) Southern blot of the samples. Lanes 1 to 16 - non-leprosy patients, 17 to 20 - healthy individuals, 21 - negative control, 22 - positive control.**

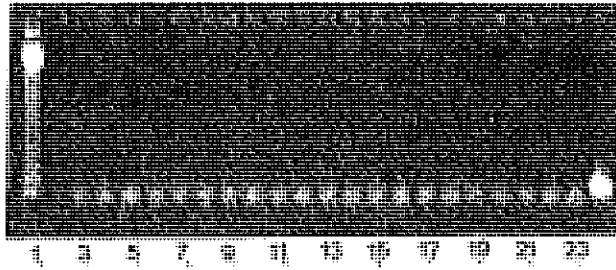
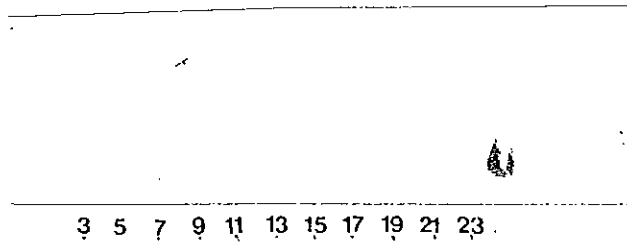
**A****B**

Fig. 10. RT/PCR for *M. leprae* in control skin biopsies. Amplification of 16S rRNA using Bio-P2 + P3 Primers. (A) Agarose gel electrophoresis. Lanes 1 - molecular weight markers, 3 to 18 - biopsies from non-leprosy patients, 19 to 22 - healthy individuals, 23 - negative control, 24 - positive control. (B) Southern blot of the above gel.

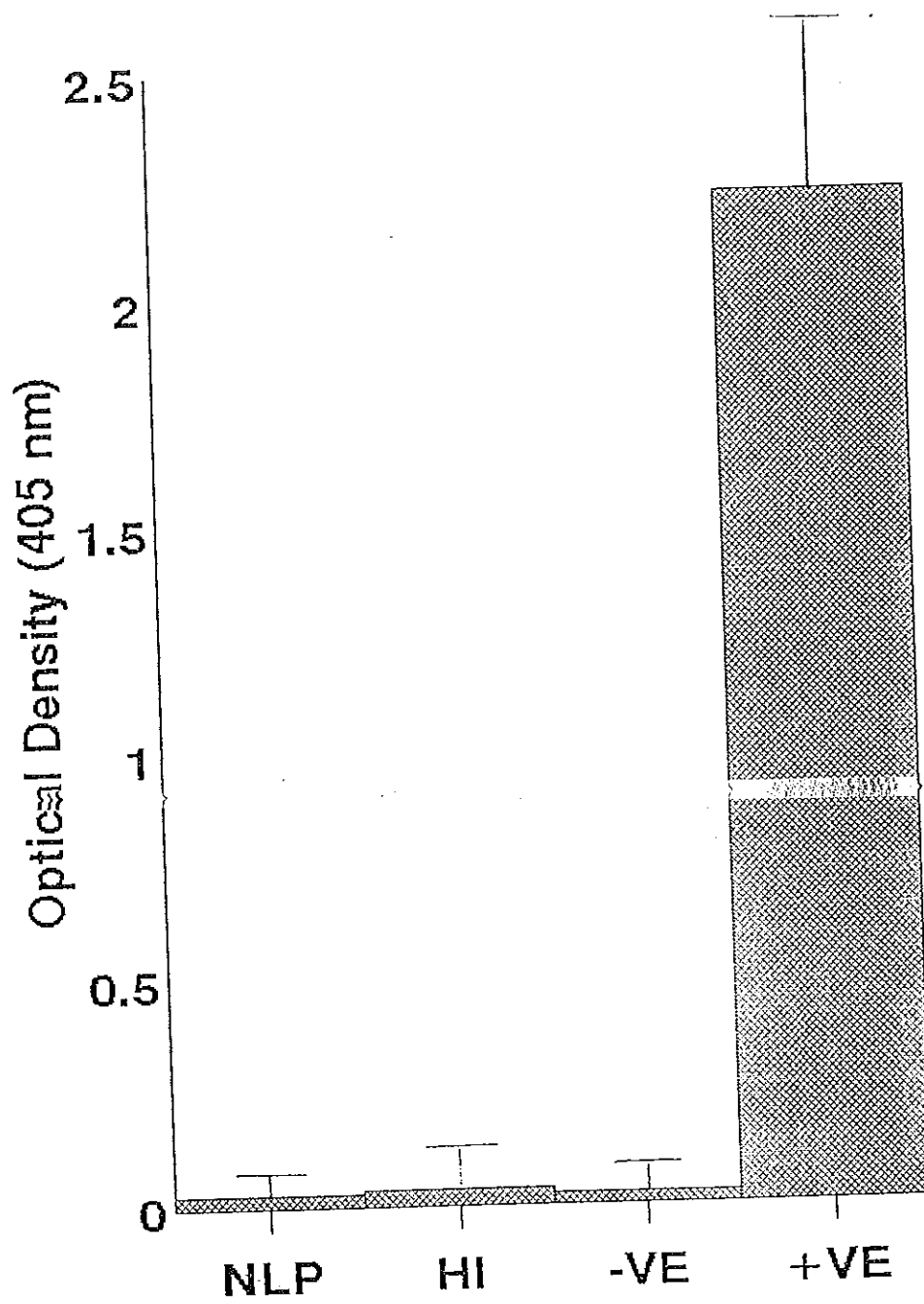


Fig. 11. ELISA-based RT/PCR products to non-leprosy patients and normal individuals. Skin biopsies were obtained from result of non-leprosy patients (NLP), healthy individuals (HI), Negative control for PCR mix (-VE), and positive control for PCR mix(+VE).

### 3.10. Comparison of microtiter plate hybridization assay with Southern blot

RT/PCR was performed on a known concentration of cDNA of *M. leprae* purified from mouse foot pad. Then the amplified products was serially diluted and aliquots of the mix was placed in the microtiter plate assay (in duplicate) or subjected to gel electrophoresis and transferred to Nylon membrane for Southern blot hybridization. Based on this experiment the microtiter plate assay was more sensitive than chemiluminescence-based Southern blot hybridization (Table 4) and was able to detect the 16S rRNA target sequences from as little as 35 pg of RT/PCR product.

Table 4. Comparison of the sensitivity of the colorimetric assay with gel electrophoresis and Southern blot hybridization of *M. leprae* 16S rRNA RT/PCR products. The RT/PCR product was serially diluted and equal concentration was subjected to gel electrophoresis, Southern blot hybridization, or the ELISA-based assay in parallel.

GEL	+	+	+	-	-	-	-	-	-
BLOT	+	+	+	+	+	-	-	-	-
ELISA	+	+	+	+	+	+	-	-	-
Conc. (pg)	5610	561	280	140	70	35	17	8	0

**Key**

GEL = Gel electrophoresis

BLOT = Southern blot

ELISA = Colorimetric microtiter assay

Conc. (pg) = Concentration in pico-gram

GEL + = Visualization of band on gel after 20 minutes staining with ethidium bromide

BLOT + = Appearance of band after 30 minutes exposure of the film

ELISA + = Visualization of yellow color after two hours incubation of the plate at dark

- = Negative

### 3.11. Detection of *M. leprae* in clinical specimens

A total of 61 biopsies from leprosy patients across the clinical spectrum were analysed using the RT/PCR assay. After normalization based on the GAPDH signal input cDNA for the 16S rRNA of *M. leprae* was performed. Positive results were obtained from biopsy specimens of all MB patients and 80% (20/25) of PB biopsies.

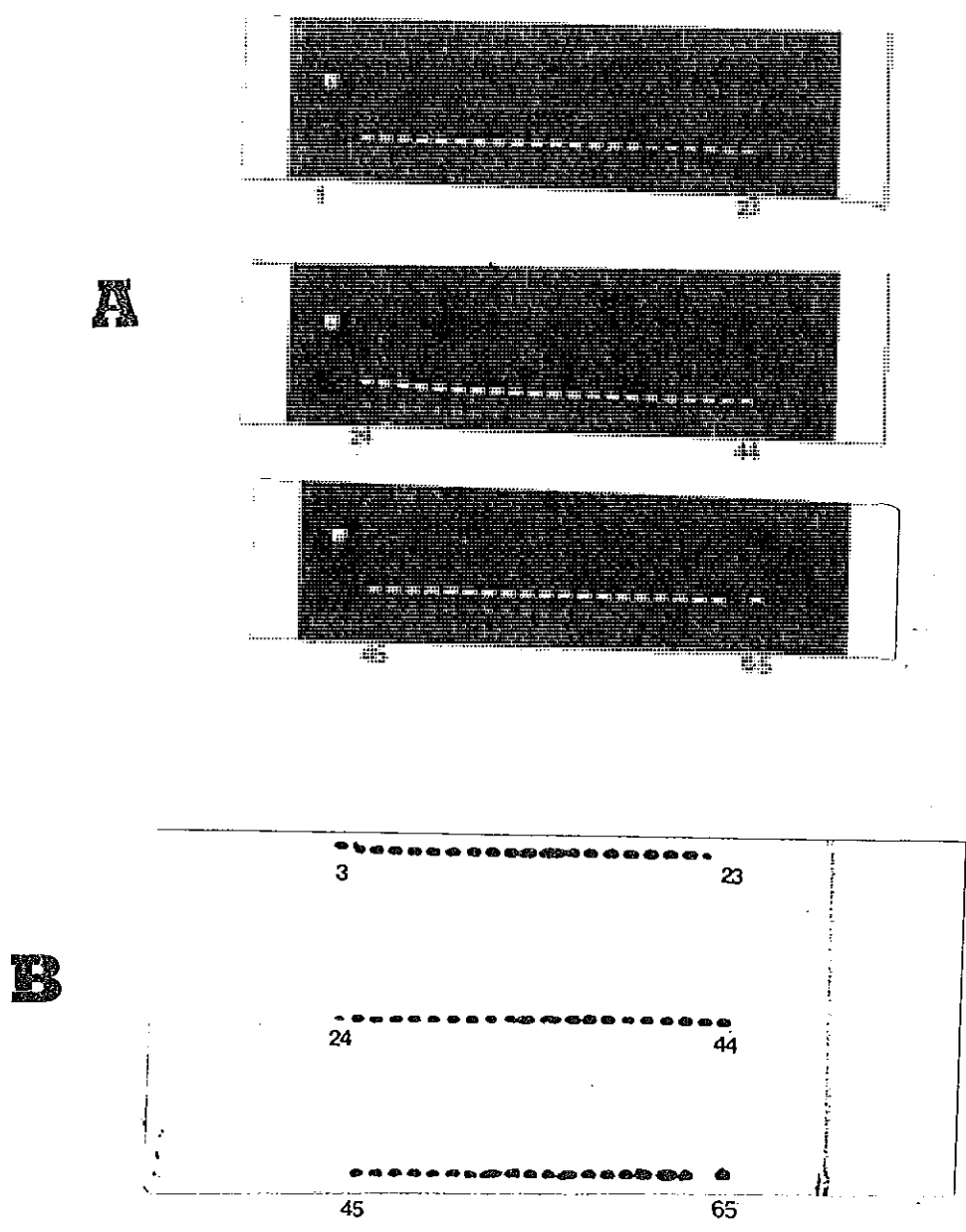


Fig. 12. PCR amplification of GAPDH from patient biopsies for 35 cycles. (A) Agarose gel electrophoresis. Lanes 1 - molecular weight markers, 3 - 27 paucibacillary leprosy, 28 - 63 multibacillary, 64 - negative control, 65 - positive control. (B) Southern blot. Lanes 3 - 27 paucibacillary leprosy, 28 - 63 multibacillary leprosy, 64 - negative control, 65 - positive control.

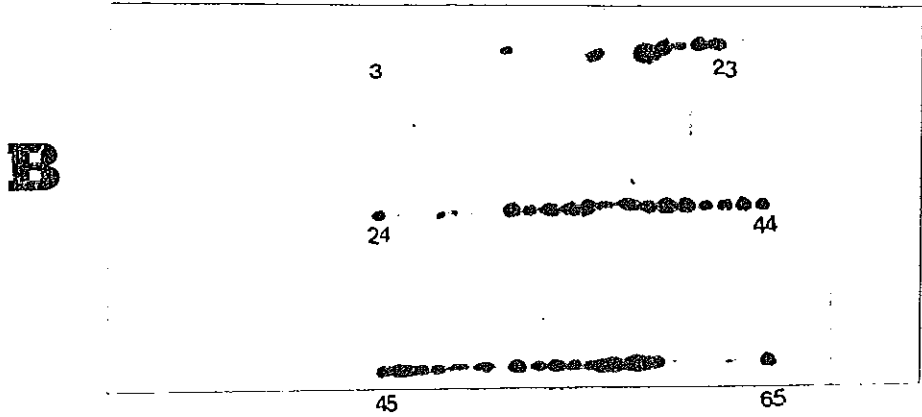
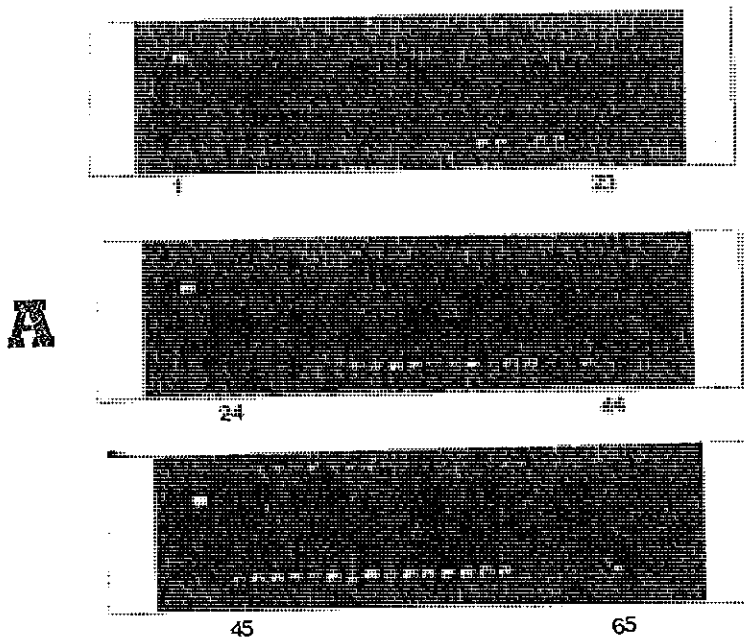


Fig. 13. RT/PCR for *M. leprae* 16S rRNA using Bio-P2 & P3 primers after 40 cycles. (A) Agarose gel analysis of the specimens. Lanes 1 - molecular weight markers, 3 - 27 paucibacillary leprosy, 28 - 63 multibacillary leprosy 64 - negative control, and 65 - positive control. (B) Southern blot of the above gel.

Table 5. Detection of *M. leprae* by ELISA-based assay for RT/PCR product.

Clinical classification	Number ELISA-based positive	Number of biopsies
Paucibacillary leprosy	36 (100%)	36
Multibacillary leprosy	20 (80%)	25
Total	56 (91.8%)	61

Table 6. Comparison between *M. lepre* RT/PCR and acid fast staining of specimens from leprosy patients and controls

Biopsy specimens	AFB +	Histology	Gel	Blot	ELISA-based positive
Leprosy patients	39/61 (63.8%)	32/42 (76.1%)	36/61 (59%)	46/61 (75.4%)	56/61 (91.8%)
Non leprosy patients	ND	ND	0	0	0
Normal individuals	ND	ND	0	0	0

#### 4. Discussion

During the last few years new diagnostic tests for the detection of *M. leprae* in clinical specimens have been introduced (Williams *et al.*, 1990., Woods and Cole, 1989., Van der Vliet *et al.*, 1993., Kurabachew, 1995). ELISA-based RT/PCR has become a popular approach for diagnosis of various infectious diseases (Nutman *et al.*, 1994). There are several thousand copies of 16S rRNA per live bacterium and thus, this approach can be quite sensitive. Furthermore, since rRNA degenerates quickly when bacteria die, its detection may indicate the presence of live bacteria. In this study an ELISA-based RT/PCR approach that uses 16S rRNA as the target sequence was developed for *M. leprae*. The assay is both sensitive and specific for *M. leprae*.

Two sets of primers for *M. leprae* 16S rRNA were used. The amplified sequence based on P1 + P3 primers is specific for *M. leprae* (Kurabachew *et al.*, 1997., Kurabachew, 1995). Another set of primers (P2 + P3) which differ by only four base pairs was also used. The P2 primer contains the shortest sequence which does not overlap with the internal probe used in both the Southern blot and the ELISA-based detection of RT/PCR products. Both sets of primers gave strong positive signals. Furthermore, we showed that the P2 and P3 primers did not detect mycobacterial species with the most closely related 16S rRNA sequences.

Optimization of the system with respect to reagent concentration, temperature, and the minimum time needed for maximum signal output was important. Since streptavidin

possesses high affinity for biotin ( $K_d = 10^{-15}$ ), and every streptavidin molecule contains four biotin binding sites, a biotin-labelled amplicon can potentially bind to the Streptavidin molecule via a strong non-covalent bond. All biotinylated RT/PCR products, and unincorporated biotinylated primers should be bound to streptavidin, since the concentration of streptavidin is always in great excess.

The temperature and buffer conditions for hybridization of the probe are based upon the melting temperature of the probe, which is determined from the G+C content and the length of the probe, as well as on the salt concentration of the hybridization buffer. As a general rule the actual hybridization temperature can range from 5 to 20°C lower than the predicted melting temperature of the probe. In this experiments, hybridization of the fluoresceinated probe to the captured RT/PCR product was maximum after one hour at room temperature. The specificity of the system is increased by lowering the salt concentration. Different concentrations of SSPE were examined with 0.1% SDS for stringency wash. When the concentration of SSPE was increased to 1X and 2X the background became progressively higher. In addition, when the concentration of SSPE is very low (0.001X and 0.005X) all fluoresceinated probe was washed away even in one minute. In contrast, 0.01X and 0.05X SSPE at room temperature for 5 minutes were optimum concentrations for the post hybridization washes.

The sensitivity of the ELISA-based detection system for the *M. leprae* 16S rRNA amplicon was evaluated by three different approaches. First, cDNA derived from a

patient biopsy was amplified by PCR, and the sensitivity of the assay was tested with various dilutions of the RT/PCR product. Sensitivity was also tested using serially-diluted *M. leprae* isolated from mouse foot pad. When the RT/PCR products were tested using the ELISA-based method, the detection limit was found to be 10 bacilli. The same test was done several times using different sources of *M. leprae*, and variation (10 - 100) was observed. The variation in sensitivity may be due to the viability of the number of bacilli in the suspension. The same sensitivity testing was done by different investigators for the same target sequence. For example, Kurabachew, (1995) and Kurabachew, *et al.* (1997) detected as few as 10-23 bacilli using chemiluminescence-based Southern blot hybridization. There is no significant difference between the later result and ours. In contrast, when equal amounts of serially diluted RT/PCR products were subjected to ELISA-based detection and Southern blot hybridization in parallel, the ELISA-based assay was more sensitive than the Southern blot hybridization assay. Furthermore, increased in sensitivity of the ELISA-based assay over the Southern blot hybridization for RT/PCR products generated from patient biopsies was also observed.

The main objectives of PCR assays to detect *M. leprae* has been to assess its usefulness as a tool to evaluate leprosy control programs, with the ultimate goal is to interrupt the transmission of *M. leprae*, reduce disease incidence and thus prevent disability. To achieve this it is necessary to detect *M. leprae* at the sub-clinical stage. Previously investigators had analyzed different target sequences and extraction procedures for simple, inexpensive, safe and sensitive detection of *M. leprae* in patient samples.

Hartskreel (1989) had reported that using specific primers for a nucleotide sequence of a gene encoding the 36 KD antigen DNA corresponding to 1 bacterium can be detected. The use of repetitive sequence (RLEP) as a PCR target DNA provides a theoretical advantage of higher sensitivity due to its presence in several copies (28) in the genomic DNA. Woods and Cole (1989) had reported that by targeting the repetitive sequence of *M. leprae* they could detect as few as 100 bacilli. Yoon *et al.* (1993) amplified the same target sequence, and determined a PCR positivity of 93.1% on the patients samples.

Targeting genomic DNA to detect live bacilli by PCR has not been well established. This method has enabled identification of *M. leprae* DNA from a bone dating from 600 AD (Rafi *et al.*, 1994). Since DNA can survive in living host tissues for long after infecting bacilli have died, and even long after their acid fast ghosts have disappeared, the application of PCR for diagnosis and evaluation of treatment is ambiguous. In contrast, targeting rRNA has attractive advantages for the detection of *M. leprae*. After cell death ribosomal RNA is destroyed more quickly than genomic DNA, the measurement of RNA is likely to correlate better with the presence of live bacteria (Katcho, 1989). Based on 16S rRNA as a target sequence, and using chemiluminescence-based Southern blot hybridization as for detection, Kurabachew (1995) found 13 positive samples out of 14 biopsies (93%). Similar results have been obtained in this study. When 61 biopsies from leprosy patients were examined 56 (91.8%) were positive using the ELISA-based detection system. Biopsies from 20 out of 25 (80%) PB patients, and 36 out 36 (100%) MB patients were positive for *M. leprae* by ELISA-based RT/PCR. The absence of

positive signals in 5 PB patients was probably due to the absence of live bacilli. Nutman *et al.* (1994) had reported that ELISA-based assay was ten times more sensitive than a chemiluminescence-based slot blot hybridization by using onchocerciasis-specific primers. Hence, it seems evident that ELISA-based RT/PCR is more sensitive than Southern blotting in detecting low levels of nucleic acid. In addition, it is inexpensive and rapid.

Despite the fact that ELISA-based assays is a technique which can be applied to the diagnosis of various infectious diseases, it has certain drawbacks. In the ELISA-based RT/PCR, extreme care must be taken to avoid false positive and false negative results. A major problem of ELISA-based RT/PCR may be contamination of samples and reagents by RNAase and carry-over of amplified products from a previous amplification of the same target. Therefore, the use of RNAase free reagents, and water is required in all experiments. Moreover, use of disposable gloves and vapor barriers pippete tips to prevent carry-over of cDNA is necessary, and each reaction must be set up in a biosafety cabinet, in a separate room, and all reagents stored separat from PCR-amplified products.

However, potential limitation concerns using pNPP as a substrate for alkaline-phosphatase. This requires an ELISA reader for quantitative detection and it is relatively difficult to visually detect weak positives. However, this problem can be solved by extending the incubation time to over 16 hours. Alkaline phosphatase is

recommended because of its rapid catalytic rate and resistance to inactivation by common laboratory reagents. Additionally, alkaline phosphatase is non-toxic and relatively stable.

Our assay had very good sensitivity combined with a background level that was clear for ELISA. Interestingly we found that, in the case of weak positive results, color developments appeared after 24 hours incubation with the substrate. The negative controls remained the same after 24 hours, but weak positives could be visualized after 24 hours incubation.

RT/PCR amplification using species-specific primers in combination with an ELISA-based detection could theoretically play a significant role in the diagnosis of leprosy at the subclinical stage. However, its use as a screening tool is limited by its expense and its requirements for sophisticated laboratory equipment such as a thermocycler. In contrast application of ELISA-based RT/PCR may be useful for diagnosis of difficult cases, such as patients with a BI of 0. It may also prove to be useful in evaluation of relapse versus late reaction. Finally it may be useful in epidemiological studies. However, more extensive examination and follow up study must be done in treated patients to evaluate its applicability.

## 5. Conclusion and recommendation

In this study an ELISA-based RT/PCR assay for the detection of *M. leprae* in patients has been developed by using 16S rRNA as the target sequence. Based on this experiments microtiter plate hybridization assay has a sensitivity that is greater than that of standard Southern blot hybridization. An assay that detects viable organisms would be a useful tool for the diagnosis of difficult cases, like AFB cases and presence of persisters after chemotherapy.

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