QUANTITATIVE DETECTION OF *Mycobacterium leprae* IN CLINICAL SPECIMEN BY THE POLYMERASE CHAIN REACTION

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Abbreviations

AMV RT = Avian Myeloblastosis Virus Reverse Transcriptase

Ag = Antigen

Ab = Antibody

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 = complementary DNA

DNA = Deoxyribonucleic acid

DTH = Delayed Type Hypersensitivity

dNTP = deoxynucleotide triphosphate

DTT = Dithiothreithol

DMSO = Dimethyl Sulphoxide

ECL = Enhanced Chemiluminescence

ENL = Erythema Nodosum Leprosum

EDTA = Ethylenediaminetetraacetic Acid.

GAPDH = Glyceraldehyde Phosphate Dehydrogenase

HSP = heat-shock protein

I = Indeterminate leprosy

LL = Lepromatous leprosy

MoAb = Monoclonal Antibody

MI = Morphological index

MB = Multibacillary leprosy

PB = Paucibacillary leprosy

PBMC = Peripheral Blood Mononuclear Cell

PGL-1 = Phenolic glycolipid-1

PHA = Phytohaemagglutinin-A

PAGE = Polyacrylamide Agarose Gel Electrophoresis

PCR = Polymerase chain reaction

pra = Proline-rich antigen

RLEP = Repetitive leprosy sequence

RFLP = Restriction Fragment Length Polymorphism

RT-PCR = reverse transcription-PCR

RNA = Ribonucleic acid

rRNA = ribosomal RNA

SDS = Sodium Dodecyl Sulphate

Taq = *Thermophillus aquaticus*

TT = Tuberculoid leprosy

ABSTRACT

Detection of *M.leprae* by PCR provides a tool for identification of organisms in clinical specimens. Genomic DNA and RNA were isolated using commercially obtained extracting solutions (DNA STAT 60™ and RNA STAT 60™). RNA was reverse transcribed to make cDNA and both genomic DNA and cDNA were subjected to PCR amplification. Products were electrophoresed through agarose gels, stained with ethidium bromide, blotted onto nylon membranes, hybridized with a 16S rRNA oligonucleotide probe and subjected to non-radioactive detection. Several sets of 16S rRNA primers were investigated. Of these, one set of primers (P2 and P3) (P2= bp # 68-90 and P3= bp # 218-239) was confirmed to be specific for *M.leprae* by testing its ability to amplify a panel of 28 potentially cross-reacting species of mycobacteria, 8 related non-mycobacterial isolates and 2 nasopharyngeal commensal organisms. In addition no amplification was observed when skin biopsies from normal individuals or patients with skin diseases other than leprosy were tested. Another set of primers (P1 and P3) (P1= bp # 9-28 and P3= bp # 218-239) was genus-specific. After optimization of the PCR condition, the sensitivity of the system was assessed using *M.leprae*-specific primers and the detection limit was 23 bacteria. This technique, therefore, appears to meet the criteria of sensitivity and specificity and may serve as a useful tool for the diagnosis of leprosy. By examining a limited number of tissues from leprosy patients, we were able to detect PCR signals in all MB (n= 5) and in 89 % (n= 9) of PB patients. We also quantitated numbers of organisms in these specimens by comparing the density of the PCR signal of samples on hybridized Southern blots to that of serial dilutions of known number of organisms.

1. INTRODUCTION

1.1. The disease

Leprosy is a chronic infectious disease of man caused by the obligate intracellular pathogen, *Mycobacterium leprae* (Job *et al*, 1971). It results in disabilities which increase in time and can be permanent. Although the bacterium has special predilection for skin and peripheral nerves, it also affects testes, eyes, liver, the mucosa of the upper respiratory tract, bone and muscles, often resulting in impairment of working capacity and seriously affecting the patients' social life (Mshana *et al.*, 1983; Narayanan, 1988; WHO, 1988; Bryceson and Pfaltzgraf, 1990).

1.2. Prevalence and distribution.

The number of leprosy cases as estimated by WHO (Nordeen, 1991) for 1966 and 1976 was 10.8 and 10.6 million respectively and a slight increase (10 - 12 million cases) was seen in the early 1980s. However, during the past 8 years the numbers of estimated and registered cases of leprosy has fallen from 10 - 12 million to 2.7 million and from 5.4 million to 1.9 million respectively. This drastic reduction has been attributed to the implementation of multidrug therapy (WHO Bulletin, 1994).

To estimate the numbers of leprosy cases in the world accurately is not

that easy. Due to the lack of a clear-cut and consistent definition and criteria for diagnosis, the enumeration of cases in many countries is incomplete. However, estimates are extrapolated from the available data from time to time (WHO, 1988). This method may overestimate or underestimate the number of estimated cases and can not be taken as a reliable method.

The only reliable method observed so far for estimating the total number of leprosy cases in particular country has been based on random sample survey. However, this method is expensive, time consuming and needs statistical support (WHO, 1988). Hence, there should be a means to develop simple, standard method to estimate the number of cases roughly in a given area so that it would be enough for planning and operational purposes.

Although leprosy once had a remarkably cosmopolitan distribution, the majority of patients are at present concentrated mainly in developing countries (WHO, 1988). Leprosy is widely distributed in Africa, Asia, South America and is still present at a very low level in parts of Eastern and Southern Europe and in small communities in USA where there is a low standard of living, poverty and crowded living conditions (Bryceson and Pfaltzgraf, 1990). Of the estimated cases, the major proportion (about 62%) is found in Asia, followed by Africa (about 34%). About 3% live in South America and the remaining 1% in the rest of the world. However, in terms of intensity of the disease in the population, i.e, mean prevalence by continent, the problem in Africa is about

three times as intense as it is in Asia (Hastings, 1985). On the other hand, leprosy has been eradicated in most of North America, Western and Northern Europe (except Iceland and in coastal areas in USA) (Bryceson and Pfaltzgraf, 1990; Nordeen *et al*, 1992).

Although there has been a sharp decline in prevalence of leprosy worldwide over the past decades, the incidence has remained unchanged in many developing countries (van Bears *et al*, 1994). According to Nordeen (1991) about 1,320 million people live in 28 countries where leprosy remains a serious health problem and it is assumed that all are at risk for contracting the disease.

At present about 1.6 billion people live in countries where leprosy is an important public health problem, with a prevalence of more than one case per thousand people (Meyer and Walsh, 1991) and about 2 to 3 million people are estimated to be handicapped as a result of having contracted leprosy (van Bears *et al.*, 1994).

1.3. Leprosy in Ethiopia

Leprosy is still a disease of major importance in Ethiopia. Except for some isolated areas (as in Mendi), the higher incidence is observed in highland regions in general (Gunderson, 1987; Berhe *et al.*, 1990) and in central and northern Ethiopia in particular. Such uneven distribution in the country may be due to differences in the nature and extent of the immune system. Although population

density can be one factor for the dissemination of the disease, different ethnic groups were shown to respond to the bacteria in different ways. A review of the country's leprosy control program for 14 years (1976-1989) showed that the cumulative national average prevalence rate was 2.6 per 1000. A significant reduction in prevalence rate was observed after the introduction of MDT. According to the All African Leprosy Rehabilitation and Training Center (ALERT) (cited in AHRI, 1993 Annual Report), the prevalence and detection rate per year in the former Shoa province, where 12.4 million people (25% of the country's total population) are estimated to live, was 2 per 1000 and 0.7 per 10000 population respectively.

1.4. Transmission

The spread of leprosy depends on the dissemination of *M.leprae* in a susceptible population. Although the portal of entry of this pathogen remains uncertain, transmission from an infected to healthy individuals through respiratory tract and prolonged skin to skin contact are believed to be the most likely routes (Meyers and Walsh, 1991). A few cases of transmission by tattooing needles (Godal and Levy, 1984) have also been recorded.

A number of reports have shown the existence of reservoirs of *M.leprae* other than man. *M.leprae*-like infections in feral armadillos and mangabey monkeys may provide a possible non-human source of M.leprae (Bloom and

Godal, 1983). However, the available epidemiological evidence favors human to human transmission as the most important source for infection (Meyers *et al.*, 1992).

Longitudinal studies among contacts of leprosy patients have clearly established that patients with multibacilliary (MB) leprosy are far more important as potential sources of transmission of the disease than are patients with paucibacilliary (PB) leprosy (Klatser *et al.*, 1993). According to WHO (1988) it has been repeatedly shown that household contacts of PB leprosy patients have a 2 fold higher risk of contracting the disease than individuals with no known household contact and a 4 to 10 fold increased risk is seen in similar contacts of MB leprosy patients.

1.5. Clinical Leprosy

The most remarkable thing about leprosy is the enormously wide variation in the way the disease affects different people. Although most people living in leprosy endemic areas are exposed to the bacilli, they never have symptoms or signs of the disease. Some individuals (about 5%), however, develop active disease (Bjune *et al.*, 1983; Reitan, 1985).

The clinical signs and symptoms of leprosy depend on the nature and extent of the host's immune response to the organism and upon the extent of bacillary multiplication (Brycesson and Pfaltzgraff,1990). Thus, leprosy is not

a single clinical entity, but rather the disease presents as a spectrum based on the immune response of the host to *M.leprae* antigens (Bloom, 1986; Nilsen *et al.*, 1986; Mukherjee and Thomas, 1991). Ridley and Jopling (1966) classified leprosy patients into a spectrum according to clinical and pathological evidence of immune responsiveness to *M. leprae*. The classification encompasses two polar forms, tuberculoid (TT) and lepromatous leprosy (LL), and intermediate borderline (BB), borderline tuberculoid (BT), borderline lepromatous (BL).

At the tuberculoid pole leprosy patients develop high levels of specific cell mediated immunity (CMI) that eventually kills and clears the bacilli from the tissue (Myrvang *et al*, 1973; Bloom *et al*; 1989). Often no bacilli can be seen in biopsies taken from infected tissues of such patients. In contrast, lepromatous leprosy patients exhibit selective cell mediated unresponsiveness *in vitro* and *in vivo* to antigens of *M. leprae* (Bloom *et al.*, 1989) and the bacilli multiply in the skin and nasal mucosa, often to extraordinarily numbers (as many as 10^9 per gram of tissue). Schwann cells also have a high load (Turk, Curtiss and de Blaquiere, 1991). The majority of patients fall somewhere between the two extremes. Several lines of evidence such as the lymphocyte transformation test (LTT), leucocyte migration inhibition tests and the lepromin tests have indicated that CMI decreases continuously from the TT to the LL pole (Reitan *et al*, 1982; Bloom and Godal, 1985; Bloom *et al*, 1989).

Leprosy patients who can not be classified under any of the above spectra

are called Indeterminate leprosy patients. Here, the lesion appears as a symptomless ill-defined hypopigmented macule and usually observed on the face, trunk or extensor surfaces of the limbs. In such patients sensation is mostly normal but sometimes it can be impaired slightly and the number of bacilli is very few. Hence, careful and continuous observation is mandatory to diagnose these patients (Brycesson and Pfaltzgraff, 1990). In spite of the fact that *M.leprae* is virtually non-toxic, nerve damage is widespread. Although the mechanisms responsible for the damage are not well established, it seems likely to be partially due to the patients' immune response towards antigens of the bacilli (Mshana *et al.*, 1983 ; Harboe, 1985). While nerve damage occurs along the whole spectrum, it differs in nature at the TT and LL ends (Bloom and Godal, 1983). Nerve damage in TT patients is severe but confined whereas, in BL patients nerve damage is less severe but the disease is more disseminated and encompasses larger nerves. Hence the destruction is of much greater consequence than in TT patients (Bloom and Godal, 1983). Nerve damage in LL, unlike TT leprosy, occurs in a very slow and progressive manner and affects primarily cutaneous nerves (Bloom and Godal, 1983). Due to lack of CMI in LL patients, *M.leprae* survive within the Schwann cells and may affect their ability to maintain the myelin sheath and the integrity of the axon resulting in slow degeneration of the axon and demyelination (Eustis-Turf *et al.*, 1986) which ultimately leads to irreversible nerve damage. The Schwann cells of

unmyelinated fibers tend to be the first to be infected.

Most forms of nerve destruction that occurs during reversal (Type 1) reactions are due to delayed type hypersensitivity (DTH) reactions against the antigens of *M.leprae* in the nerves. Erythema Nodosum Leprosum (ENL) (Type 2 reaction) is considered as a classical example of an immune complex mediated disease that occurs mainly in patients with LL (Harboe, 1985) and in some BL patients (Mshana, 1982).

1.6. The leprosy bacillus

The serious study of leprosy dates back to the work of Danielssen and Boeck in 1847 and the bacillus was identified for the first time from a skin lesion in Norway (Bergen) by Armauer Hansen in 1873 (Hastings, 1985; Harboe, 1983). Despite the fact that *M.leprae* was one of the first infectious organisms identified by man, our knowledge concerning the basic biology of the bacteria, including its metabolism and chemical structure, has shown little progress. This is partially due to inability to culture this organism *in vitro* (Clark-Curtiss *et al.*, 1985; Estrada *et al.*, 1989; Hartskeerl *et al.*, 1989). The discovery of experimental animals like the nine-banded armadillo (Kircheimer and Storrs, 1971) and mice (Shepard, 1960, Closs *et al.*, 1979) as models of leprosy led, to some extent, to major breakthroughs in leprosy research. Nevertheless the limitation of working *in vivo* and the very slow growth rate renders classical

genetic analysis almost impossible (Honore *et al.*, 1993).

In spite of some atypical features, taxonomically *M.leprae* is classified under the order Actinomycetales and of the family Mycobacteriaceae (Rees, 1985). It is a non-spore forming Gram positive rod-shaped bacillus measuring 1.8 μm by 0.3 μm with a parallel sides and rounded ends (Ridley, 1988) occurring commonly in clumps or globi. The cell wall of *M.leprae*, which comprises about 60% lipid by weight, is a complex protective structure. It is 20nm thick and provides survival advantage for the bacilli in adverse conditions, especially during its protracted period of generation and establishment inside the host's phagocytic cells (Hastings, 1985). *M.leprae*'s intracellular location, lipid-rich cell wall and protective capsule also protect it from attack by antibody and complement (Young *et al.*, 1990).

M.leprae has a low optimum growth temperature and replicates preferentially in cooler sites in the human body and this is shown through the studies on experimental animals such as mice (Shepard, 1960, 1965) and armadillos (Kircheimer and Storrs, 1971) which have revealed that the optimum temperature for multiplication is 30 °C. Significant reduction is observed at a temperature of 36 °C (Rees, 1985). The doubling time has been estimated to be about 13 days in mice (Shepard, 1965).

Like other mycobacterial species *M.leprae* exhibits the property of acid-fastness (described by Ehrlich in 1882). The mechanism for this staining is still

not understood in molecular terms, however, it is assumed that lipoidal components, particularly the mycolic acid esters which forms part of the bacterial wall, play an important role (Reitan, 1985).

1.7. Molecular biology of *M.leprae*

Rapid progress in understanding the molecular biology of *M.leprae* and other mycobacteria has been made in the last decade. The availability of large numbers of *M.leprae* from infected armadillo tissue has provided access to *M.leprae* nucleic acids. One of the major areas of impact of molecular biology on the study of *M.leprae* is cloning. The construction of a lambda gt11 library (Young and Davis, 1985) and a cosmid library of M.leprae (Clark-Curtiss *et al.*,1985; Eiglmeier *et al.*, 1993) were important landmarks in identifying antigens that are important to the immunopathology of leprosy.

Several protein antigens of mycobacteria have been expressed and then immunologically characterized using molecular techniques. One of the relevant antigens that has received great attention is the 65KD antigen, which belongs to a family of heat-shock proteins (Hance *et al.*, 1989; Watson, 1989) and has been shown to be a highly conserved protein. In addition, many proteins like the 70, 65, 36, 28, and 18 KDa antigens have been well characterized using monoclonal antibody (MoAb) techniques and the epitopes which evoke B and T cell responses to these antigens were identified (Watson, 1989).

A prominent landmark in the history of molecular biology of *M.leprae* is the genome project which was begun by Stewart Cole and his colleagues in Paris (Honore *et al.*, 1993). Initial results of the genome project provided informations about the genome of *M.leprae* regarding specific localization of genes and partial sequences (Eiglmeir *et al.*, 1993). The establishment of an ordered collection of overlapping clones, which collectively account for 2.8 Mb of DNA coinciding with the complete chromosome of *M.leprae,* has provided a vital resource for leprosy research (Eiglmeir *et al.*, 1993). *M.leprae* has a G+C content of 56% (Williams, *et al.*, 1990; Colston, 1993). This is relatively lower than other mycobacteria (60-67%) and has raised a debate about its taxonomic position that has been resolved by cloning and sequencing the 16S rRNA.

The 16S ribosomal RNA (rRNA) gene is the most convenient gene for studying phylogenetic relationships (Colston, 1993) and for developing methods for species identification (Cox *et al.*, 1991). This is due to its conserved nature and presence in all free-living organisms (Cox *et al.*, 1991; van der Vliet *et al.*, 1993). Comparative analysis of 16S rRNA sequences has revealed regions of both highly conserved and variable sequences (Boddinghaus et al., 1990; Rogall *et al.*,1990). The conservation of sequences allows the alignment of sequences from different mycobacteria and thereby demonstrates the taxonomic and phylogenetic relationship between organisms by showing the extent of variation within these sequences (Colston, 1993). Study of 16S rRNA sequences has made

it clear that the division between the slow-growing and fast-growing is a true phylogenetic division. Information about nucleic acid sequences also plays a role in the identification of species. For instance, by identifying species-specific 16S rRNA sequences it is possible to rapidly differentiate *M.tuberculosis* and *M.avium* (Colston and Lamb, 1989). The nucleotide sequence of 16S rRNA appears in Fig. 1 and its alignment with the sequence of related organisms appear in Fig. 2. Geographically distinct *M.leprae* isolates from humans, armadillo and mangabey monkey showed lack of strain differences by restriction fragment length polymorphism (RFLP) analysis (Williams *et al.*, 1990). It has, however, been suggested that it might be possible to detect potential strain differences within *M.leprae* isolates using rRNA sequences specific for *M.leprae* (van der Vliet *et al.*, 1993)

1.8. Detection of *M.leprae*.

Detection of mycobacteria in clinical specimens remains a problem and different approaches have been used, each having its own merits and limitations. Identification of acid fast organisms by microscopy of slit skin smears is the most widely-used test to diagnose leprosy (Ohman, 1986). This method is rapid and relatively cost effective. However, it is not sensitive (Klatser et al., 1993). In addition, because other species of mycobacteria share the property of acid fastness, the method lacks specificity. Several lines of evidence have shown that

slit skin smears are negative in TT and often in BT leprosy because the number of bacilli must exceed 10^4 per gram of skin to get a positive result (Hance *et al.*, 1989; Bryceson and Pfaltzgraff, 1990).

Bacillary Index (**BI**), the density of bacilli in slit skin smears, is determined by microscopic examination of acid-fast bacteria. The classification is based on the number of bacilli that can be seen in an average field of vision (100 X oil immersion) as indicated in Table **1**. TT and BT patients usually have bacillary indices (**BIs**) that do not exceed 1+ and are grouped as PB patients while the rest (BB, BL and LL) have a BI of 2+ or more and are grouped as MB patients. Using this technique it is difficult to detect bacteria from virtually all PB patients and some MB patients and hence the method is a poor diagnostic tool. In addition this technique needs well experienced technicians. Otherwise, erroneous classification of patients may occur i.e, PB patients may be classified as MB and vice-versa. The risk is most significant if MB patients are regarded as PB patients and get inadequate treatment. In these cases the individual may not be cured of the disease, the infection may spread to others, and the reputation of MDT will be put in a dangerous position (Becx-Bleumink, 1992).

The morphological index (**MI**) is widely used to assess viability of *M.leprae*. It is defined as the proportion or percentage of regularly stained bacteria of the total scored (Rees, 1985). The MI is undertaken on the same stained slit-skin smears used to measure the BI. Bacilli that are uniformly stained

and not superimposed are regarded as viable. Unevenly stained, but not yet granular, bacilli are recorded as fragmented and those that appear as granules are recorded as granular and considered non-viable (Leiker, 1971). However, the morphology of the bacteria can be affected considerably by different staining techniques. Ridley and Ridley (1971) confirmed that heating in carbol-fusion raises the MI as a result of redistribution of acid-fast material in the body of *M.leprae.*

Experimental infection of animals (e.g. armadillos and mouse foot pad) is currently the best method for determining the viability of M.leprae and for assessing the efficacy of chemotherapy. However, this method is expensive, time-consuming and is not quantitative (Hartskeerl *et al.*, 1990; Jamil *et al.*, 1993). In addition there are very few facilities available where the mouse footpad assay can be performed.

Serological tests are also used to study *M.leprae* infection. The method depends on detection of antibodies to species-specific *M.leprae* antigens. The presence of IgM antibodies to PGL-I was detected in 23.1% (3 out of 13) of the tested leprosy patients as measured by ELISA (van Beers *et al.*, 1994). All sera obtained from MB patients showed positive results. Hence there is a strong correlation between levels of antibody and the amount of bacteria (van Beers et al., 1994). A higher antibody titre is observed in sera of LL patients and a decrease is seen as one goes down to TT (Brycesson and Pfaltzgraff, 1990).

BI	Number of bacilli
6	> 1000 in an average field
5	100-1000 in an average field
4	10-100 in an average field
3	1-10 in an average field
2	1-10 in ten fields
1	1-10 in one hundred fields
0	no bacilli found

Table 1. Numerical representation of bacterial load in skin biopsy samples.

Although serology may provide good information on the extent of transmission of the infection in a population, its diagnostic usefulness has been found to be limited for many reasons. Many studies showed that some cases of leprosy remain seronegative (van Beers *et al.*, 1994). In addition, infected individuals may remain seronegative because triggering the immune response of some individuals may require high loads of bacilli (Rinke de Wit *et al.*, 1992). In contrast, individuals can be seropositive due to past infection and thus the test may not give any information about the present bacteriological status.

Direct detection of *M.leprae* or one of its constituent antigens is useful to detect active infection. One of the antigens of choice is phenolic glycolipid-1 (PGL-1). This is because it is specific and abundant. PGL-1 is found in the urine of leprosy patients and its amount can be easily measured by concentrating the urinary bacterial components. Mahon et al. (1991) measured PGL-1 of *M.leprae* in the urine of 179 leprosy patients using an ELISA method and observed correlations between PGL-1 levels and disease spectra. The highest antigen level was observed in LL patients and a progressive decrease in amount was seen as the disease classification proceeded to the tuberculoid end of the clinical spectrum. They were able to detect the antigen in nearly all (92%) of the LL patients, in more than half (56%) of BL and in 18% of BT patients. Detection of PGL-1 is simple, suitable for diagnosis (because it shows active

infection) and not expensive. However, the antigen cannot be readily detected in PB patients, especially in the tuberculoid and hence the test lacks the required sensitivity.

Monoclonal antibodies (MoAbs) have been used to rapidly and precisely identify *M.leprae*. However, these methods may lack the required sensitivity and specificity. For example, Naafs *et al.* (1988) showed that a monoclonal antibody raised against the 65KDa antigen of *M.leprae* reacted with normal components found in host cell's cytoplasm. The two possible explanations for this are that either the antibody reacts with any partially identical antigenic determinant, or that it reacts with fully identical determinants of different origin, i.e. human heat shock proteins.

1.9. PCR detection of *M.leprae*

Recently there has been a move towards the detection and identification of microorganisms using molecular techniques. These are vital for detecting microbes such as *M.leprae* that do not grow in artificial media (Waters, 1993) or are slow-growing. Mycobacteria can be detected by DNA or RNA hybridization using labelled probes specific for the DNA sequence of interest. This method is rapid as well as reliable but not used for routine work. In addition, due to the presence of insufficient amount of mycobacterial DNA in many clinical samples, it is often impossible to get a positive signal using this

technique and so the method is not as sensitive as is needed (Hance *et al.*, 1989). Nevertheless, the use of DNA and RNA probes paved the way to the application of the polymerase chain reaction (PCR). Hartskeerl *et al.* (1989) used primers specific for the 36KDa antigen of *M.leprae* to obtain a better sensitivity than DNA-hybridization in clinical samples. The PCR is proving useful in the early and rapid detection of mycobacterial infections in clinical specimens (Pao *et al.*, 1990).

PCR is an *in vitro* method which involves a primer-directed enzymatic amplification of a specific DNA sequence of interest. This procedure generally depends on the presence of two oligonucleotide sequences that flank the DNA segment to be amplified. Each cycle of PCR involves repeated cycle of heat denaturation, annealing the primers to their complementary sequences within the target DNA and extension of the annealed primers with DNA polymerase (Ehrlich *et al.*,1988; Bell, 1989). The end product is then denatured again and becomes a template for the next cycle resulting in the exponential accumulation of specific target fragments to a magnitude of approximately 2^n (where n is the number of cycles).

Several DNA amplification tests have been developed for detection of *M.leprae* in infected tissue. The differences lie in extraction and choice of the target sequences. The specificity of the technique is greatly influenced by the target sequence chosen. Researchers have tried to amplify *M.leprae* DNA by

PCR using different target sequences such as the 18 KDa antigen (Williams et al., 1990), the 36 KDa proline-rich antigen (pra) (Hartskeerl *et al.*, 1989; de Wit *et al.*, 1993), the 65 KDa antigen, a repetitive sequence (RLEP) (Woods and Cole, 1989) and rRNA sequences (Cox *et al.*, 1991; Arnoldi *et al.*, 1992).

PCR is more sensitive than microscopic examination in detecting *M.leprae*. Amplification using different base pair segments of the gene coding for the 65 KDa antigen (Hance *et al.*, 1989) and 36 KDa antigen (Hartskeerl *et al.*, 1989) have shown that very few bacteria (< 100) can be detected. A study made by Yoon *et al* (1993) has also shown that amplification of *M.leprae* DNA by PCR using primers amplifying the 372 base pair of the repetitive sequence (RLEP) gave a positive result in about 61% of biopsies from leprosy patients negative for AFB, thus indicating that PCR is a useful tool for laboratory diagnosis.

The use of repetitive leprosy sequence (RLEP) as a PCR target DNA provides a theoretical advantage of higher sensitivity over the first three targets (18KDa, 36KDa and 65KDa antigens) due to its presence at multiple sites in the genomic DNA (Yoon *et al.*, 1993). Woods and Cole (1990) showed the existence of at least 28 copies of RLEP in *M.leprae* and PCR products targeting the repetitive sequences appeared 6 cycles earlier than those targeting a single copy sequence. They compared RLEP primers with primers targeting groEL (65KDa) genes. When DNA concentrations of the PCR products for these

samples were measured, the difference was 30 fold i.e. samples that contained RLEP primers had much more DNA than samples containing primers amplifying groEL genes. Although Woods and Cole (1990) did not determine how many bacteria could be detected using RLEP primers, they suggested that one bacillus might give a signal. Using these primers Yoon *et al.* (1993) obtained a positive result from samples containing one AFB, whereas primers which amplify single gene sequences, most often, require 10-100 AFB to generate a signal (Hartskeerl *et al.*, 1989).

The 16S rRNA in particular is an appealing target. The presence of thousands of RNA copies per leprosy bacillus should theoretically make this target more sensitive compared to other targets (Arnoldi *et al.*, 1992). Using this sensitive and specific procedure, it may be possible to readily detect very small number of organisms, as few as one bacteria, in test specimens.

Intact RNA is present in living cells that show active metabolism and may serve as an indicator of mycobacterial viability. RNA is more prone to degradation after cell death than other cell components such as genomic DNA. The observation that mycobacterial cell degradation is accompanied by the disappearance of ribosomes suggests that detection of RNA by reverse transcriptase-PCR (RT-PCR) may be useful to assess viability (Jamil et al., 1993; van der Vliet *et al.*, 1994).

1.10. Objectives of the study

In recent years mycobacterial infections have gained increasing clinical importance. Despite major advances in new and rapid diagnostic tests, many of the methods need further development and prospective evaluation. This is because nearly all methods suffer from the lack of either the required sensitivity or specificity or both.

The recent development of PCR has brought advances in molecular biology and has led to the development of sensitive, specific, and rapid tests to identify bacilli in clinical specimens. Many target sequences for PCR of *M.leprae* DNA have been tried, most of which are found in single copy number. The target sequence (RLEP) is present in several copies (28) in the genomic DNA, and has been adopted by WHO as the standard for the diagnosis of *M.leprae*. Most reports have shown that PCR provides 100% specificity, nevertheless, most of the PCR methods so far used have not been adequately evaluated for detecting bacteria in clinical samples. For example, greater specificity is obtained using primers specific for RLEP, but not all PB patients yield a positive result. To solve this problem, if possible, a protocol using target sequences with higher sensitivity is needed.

The 16S rRNA of *M.leprae* contains sequence information that enables mycobacterial identification at the species level. In addition the high copy number (10^3-10^4) of rRNA in every free-living cell should make its detection

easier than those which occurs in a single or low copy number. Theoretically a single bacterium can be detected using this target and it may become easier to diagnose PB patients and, to some extent, subclinical infections. This thesis work was based and carried out on this premise. The main objectives were:

1. to develop a PCR assay to detect *M.leprae* using 16S rRNA as the target.
2. to test its species-specificity and sensitivity.
3. to detect and quantitate *M.leprae* in clinical specimens using RT-PCR.

2. MATERIALS AND METHODS.

2.1. Biopsy specimens:

Skin biopsies (4-6 mm punch) from treated and untreated leprosy patients seen at the ALERT (All African Leprosy Rehabilitation and Training Center) hospital were taken after obtaining informed consent. The sample was cut into two approximately equal parts and snap frozen in liquid nitrogen. One half was used for cryoembedding and the other half stored in -80 °C until used for PCR. Classification was done clinically and histopathologically according to Ridley and Jopling scale as Indeterminate, TT, BT, BB, BL, LL.

2.2. Isolation and counting of mycobacteria:

Briefly, fresh biopsy samples were taken and dispersed using a 15 ml pyrex homogenizer (VWR Scientific Co.,England). Then a known amount of bacteria ($5x10^4$ bacteria) were taken with the help of a calibrated loop and diluted with distilled water containing 0.2% bovine serum albumin (BSA). Volumes of the diluted suspensions were spread over 8 mm diameter circles scored on clean microscopic slides. Fixation was done by letting the slide air dry, treating it with 40% formalin for 15 min. and heating on a hot plate (60^0C) for 5 min. The slide was then stained by the Ziehl-Neelsen method i.e. it was covered with carbol-fuchsin, heated until steaming, left for 15 min. and washed with tap water for 2

min. 20% of sulfuric acid (decolourizer) was then poured onto the slide for one minute and washed again with tap water for 2 min. Brilliant green was then added to the slide and left for 2 min., washed with tap water for two min. and allowed to dry. The slide was examined under oil immersion and bacterial count was done for 8 high power fields spaced 1 mm apart across the diameter. The number of bacteria in the original suspension was calculated by taking into account of the diameter of the high power field, the film that was counted and the dilution. The percentage of solid bacilli, the MI, was calculated after examining one hundred bacilli lying separately. Only perfectly rod-shaped and uniformly-stained bacilli were considered as live bacilli.

2.3. Nucleic acids extraction:

2.3.1. DNA STAT 60 ™ and RNA STAT 60 ™: Biopsy specimens were embedded with cryoembedding medium, sprayed with cryospray (both from Bright Instrument Co Ltd, England) and cut to 5 μm thickness (40 slices) using disposable microtome blades in a 1720 kryostat (Leitz, Germany). To avoid cross-contamination of samples during processing, blades were used only once and the instrument was cleaned with alcohol before processing the next sample. The sample was then homogenized using 0.1 mm diameter glass beads (Biospec products, Barteesville, U.S.A.) in 1 ml **DNA STAT 60™** (to extract DNA) or

RNA STAT 60™ (to extract RNA) for 1 min. and sonicated for 5 min. at 60°C using a sonicator (Elma®, Germany) at a frequency of 35 KHz. Then 200 μl of chloroform was added to the homogenized sample, and it was mixed and centrifuged at 12,000g for 15 min. at 4 °C. The aqueous phase containing nucleic acids was then mixed with 500 μl isopropanol to precipitate RNA or DNA, left at room temperature for 5 min. and centrifuged at 12,000g for 10 min. After decanting the supernatant, the DNA or RNA pellets were washed with 1 ml alcohol (75%) and centrifuged at 7,500g for 5 min. Finally, most of the alcohol was removed using a micro pipette and the DNA pellets were dried using a speed vac concentrator (Savant Instruments Inc., NY). RNA pellets were obtained by letting the sample air dry. The DNA obtained from **DNA STAT 60™** was used directly as a template in PCR reaction, whereas RNA (obtained using **RNA STAT 60 ™**) was reverse transcribed to make complementary DNA (cDNA). To avoid degradation by contaminating RNAses, RNA was treated with commercially obtained RNAse-free water, chemical reagents, glassware and tubes, and every procedure was done wearing gloves.

2.3.2. Freeze/thaw method: Samples containing bacteria were overlaid with about 45 μl mineral oil (Sigma) to avoid evaporation and subjected to a series of heat/cold shocks, alternately boiled in a heating block (Hybaid Omni Gene, Temperature cycler; U.K.) at 95°C for 2 min and then snap frozen in liquid

nitrogen for 2 min. This step was repeated 5 times before the sample was added to the PCR reaction.

2.4. Complementary DNA (cDNA) synthesis:

RNA was transcribed into cDNA in a total volume of 20 μl containing 50mM Tris-HCl; 8 mM $MgCl_2$; 30 mM KCl; pH 8.5 (20 °C) (Boehringer Mannheim), 6mM dithiothreithol (DTT) (Stratagene), 1mM dNTP mix (0.25 mM dATP, 0.25 mM dTTP, 0.25 mM dGTP and 0.25 mM dCTP) from Boehringer Mannheim, primers (0.4 μg of synthetic oligo $(dT)_{15}$ primer (Promega) and/or 20 pmol (each) of random hexamers and/or antisense primer (P3) used in the PCR reaction), 0.8U of RNasin and 20U of AMV reverse transcriptase (AMV RT) (both from Stratagene). The reaction mix was incubated for 50 minutes at 42°C. It was then heated for 5 minutes at 95°C to inactivate the RT, quickly cooled on ice and finally diluted with double distilled deionized water to make the total volume 100 μl. It was then stored at -20 until used for PCR.

2.5. PCR amplification: PCR was performed in a total volume of 100μl using a thermal cycler (Hybaid, Omni Gene, UK). The initial reaction mixture (Boehringer Mannheim) contained 10 mM Tris HCl (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl; 5 mM deoxynucleoside triphosphate (dATP, dCTP, dGTP and dUTP), 100 pmol each of the sense primer (forward primer)and anti-sense primer

(backward primer) (Johns Hopkins University; School of Medicine, U.S.A.) and 2.5 U of heat stable Taq DNA polymerase (Boehringer Mannheim) derived from *Thermophillus aquaticus*. After adding 10 μl of the template (either from the prepared cDNA or genomic DNA), the mixture was overlaid with about 45 μl of mineral oil (Sigma). The thermal cycling profile for 16S rRNA primers involved 40 cycles of heat denaturation at 94°C for 2 min, primer annealing at 58°C for 2 min and primer extension at 72°C for 3 min. After the 40th cycle, extension was continued for a further 15 min at 72°C.

2.6. Optimization of buffers, temperatures and additives:

Reaction buffers and conditions were altered to optimize the system. Different buffers, adjuncts and temperatures were tested. Twelve different buffers which varied in pH, magnesium chloride ($MgCl_2$), and potassium chloride (KCl) concentration but with equivalent Tris-HCl concentrations were obtained commercially from Stratagene (shown in Table 3) and one buffer from Boehringer Mannheim were tested. Three different thermal profiles were also tried by changing the annealing temperature (denaturation and extension temperatures remained the same for all PCR conditions). The annealing temperatures were 55°C, 57°C and 60 °C. In addition six different adjuncts obtained from Stratagene (5 μg BSA, 5 % formamide, 10 % dimethyl sulphoxide (DMSO), 15 % glycerol, 20 mM $(NH_4)_2$ SO_4 and 1 U Perfect Match^R DNA

Polymerase enhancer a proprietory adjunct made by Stratagene and enhances the specificity and yield of PCR) were tested in different tubes that contained similar reaction mixtures and were treated identically.

2.7. Primers:

All primers were purchased from the DNA analysis facility at Johns Hopkins University School of Medicine in Baltimore MD. Sequences of primers used appear in Table 3. Primers P1, P3 and P5 were published by Arnoldi *et al.* (1992) and P2 and P4 by Cox *et al.* (1991).

2.8. Agarose gel electrophoresis:

Equal aliquots (most often 15 μl) of the PCR products were loaded on an agarose gel (usually 1.5% and rarely 1.8 when better separation was needed) and electrophoresed at the desired voltage for varying length of time as required (voltage applied varies inversely with time) in Tris acetate EDTA (TAE) buffer (1x TAE = 4.84 g Tris base, 1.14 ml glacial acetic acid and 0.744 g Na_2 EDTA.2 H_2O dissolved in a liter of double distilled water, pH = 8.5). The DNA was visualized using a UV transilluminator (UVP, INC. San Gabriel, U.S.A.) after staining with ethidium bromide (EtBr) (0.5 μg/ml) for about 20 min. and

10 mM tris-HCl	$MgCl_2$	25 mM KCl	75mM KCl
pH 8.3	1.5 mM	Buffer 1	Buffer 2
pH 8.3	3.5 mM	Buffer 3	Buffer 4
pH 8.8	1.5 mM	Buffer 5	Buffer 6
pH 8.8	3.5 mM	Buffer 7	Buffer 8
pH 9.2	1.5 mM	Buffer 9	Buffer 10
pH 9.2	3.5 mM	Buffer 11	Buffer 12

Table 2. Concentrations of various buffer components obtained from Stratagene. (Opti-Prime™ PCR Optimization Kit).

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'
16S rRNA (P1)	S	9-28	5' AGA GTT TGA TCC TGG CTC AG 3'
16S rRNA (P2)	S	68-90	5' CGG AAA GGT CTC TAA AAA ATC TT 3'
16S rRNA (P)	P	85-112	5' CGC CAC TCG AGT ATC TCT AAA AAA GAT T 3'
16S rRNA (P3)	A	218-239	5' CAT CCT GCA CCG CAA AAA GCT T 3'
16S rRNA (P4)	A	452-472	5' GAA CCC GGA CCT TCG TCG ATG 3'
16S rRNA (P5)	A	910-929	5' CCG TCA ATT CCT TTC AGT TT 3'

Table 3. Primers and probe used in studies.

Key: A = antisense
S = sense
P = probe used for hybridization

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          P1---------------------------------▶
1 TTGTTTGGAG AGTTTGATCC TGGCTCAGGA CGAACGCTGG CGGCGTGCTT
                        P2---------------------------------------▶
51 AACACATGCA AGTCGAACGG AAAGGTCTCT AAAAAATCTT TTTTAGAGAT
                                                - - - - - - - - - - - - - - -

101 ACTCGAGTGG CGAACGGGTG AGTAACACGT GGGTAATCTG CCCTGCACTT
    - - - - - - - - - - - - -Probe
151 CAGGGATAAG CTTGGGAAAC TGGGTCTAAT ACCGGATAGG ACTTCAAGGC
                                ◀---------------------------------------P3
201 GCATGTCTTG TGGTGGAAAG CTTTTTGCGG TGCAGGATGG GCCCGCGGCC

251 TATCAGCTTG TTGGTGGGGT GACGGCCTAC CAAGGCGACG ACGGGTAGCC
301 GGCCTGAGAG GGTGTCCGGC CACACTGGGA CTGAGATACG GCCCAGACTC
351 CTACGGGAGG CAGCAGTGGG GAATATTGCA CAATGGGCGC AAGCCTGATG
401 CAGCGACGCC GCGTGGGGGA TGACGGCCTT CGGGTTGTAA ACCTCTTTCA
      ◀---------------------------------------- P4
451 CCATCGACGA AGGTCTGGGT TTTCTCGGAT TGACGGTAGG TGGAGAAGAA

501 GCACCGGCCA ACTACGTGCC AGCAGCCGCG GTAATACGTA GGGTGCGAGC
551 GTTGTCCGGA ATTACTGGGC GTAAAGAGCT CGTAGGTGGT TTGTCGCGTT
601 GTTCGTGAAA TCTCACGGCT TAACTGTGAG CGTGCGGGCG ATACGGGCAG
651 ACTAGAGTAC TGCAGGGGAG ACTGGAATTC CTGGTGTAGC GGTGGAATGC
701 GCAGATATCA GGAGGAACAC CAGTGGCGAA GGCGGGTCTC TGGGCAGTAA

751 CTGACGCTGA GGAGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
801 GTAGTCCACG CCGTAAACGG TGGGTACTAG GTGTGGGTTT CCTTCCTTGG
851 GATCCGTGCC GTAGCTAACG CATTAAGTAC CCCGCCTGGG GAGTACGGCC
                ◀----------------------------------------P5
901 GCAAGGCTAA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGCGGAG
951 CATGTGGATT AATTCGATGC AACGCGAAGA ACCTTACCTG GGTTTGACAT

1001 GCACAGGATG CGTCTAGAGA TAGGCACTCC CTTGTGGCCT GTGTGCAGGT
1051 GGTGCATGGC TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG
1101 CAACGAGCGC AACCCTTGTC TCATGTTGCC AGCACGTAAT GGTGGGGACT
1151 CGTGAGAGAC TGCCGCGGTC AACTCGGAGG AAGGTGGGGA TGACGTCAAG
1201 TCATCATGCC CCTTATGTCC AGGGCTTCAC ACATGCTACA ATGGCCGGTA

1251 CAAAGGGCTG CGATGCCGCA AGGTTAAGCG AATCCTTTTA AAGCCGGTCT
1301 CAGTTCGGAT CGGGGTCTGC AACTCGACCC CGTGAAGTCG GAGTCGCTAG
1351 TAATCGCAGA TCAGCAACGC TGCGGTGAAT ACGTTCCCGG GCCTTGTACA
1401 CACCGCCCGT CACGTCATGA AAGTCGGTAA CACCCGAAGC CAGTGGCCTA

1451 ACCCTCGGGA GGGAGCTGTC CAAGGTGGGA TCGGCGATTG GGACGAAGTC
1501 GTAACAAGGT AGCCGTACCG GAAGGTGCGG CTGGATCACC TCCTTTCT
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Fig.1. Nucleic acid sequence of *M.leprae* 16S ribosomal RNA (Liesack *et al.*, 1990). The underlined sequence shows the locations of oligonucleotides used in experiments.

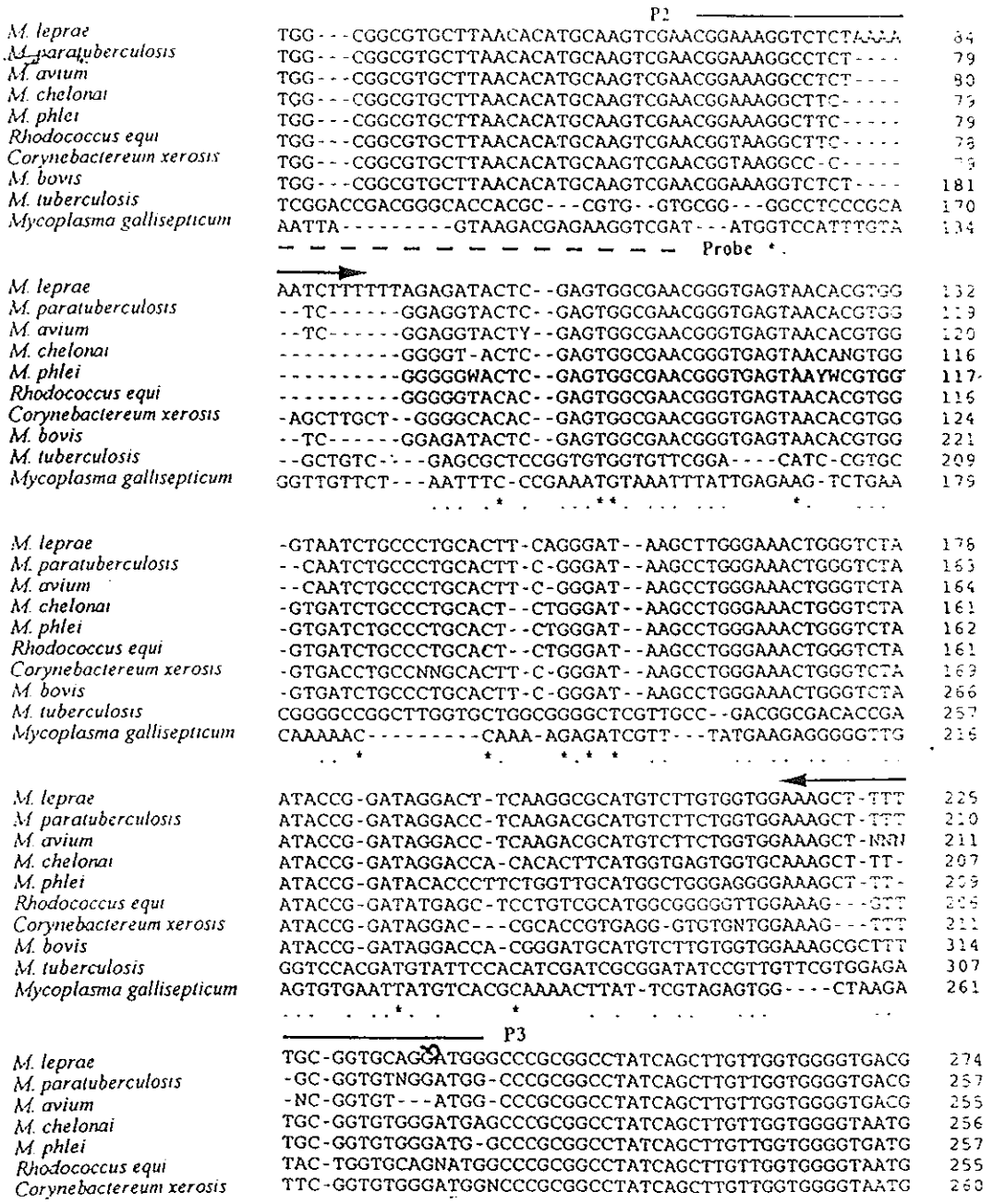

Fig. 2. Alignment of 16S rRNA coding sequences of some mycobacteria and related non-mycobacterial species.

2.9. Probe labeling:

Labelling was done following the procedure provided by the manufacturer of the ECL ™ 3'- oligolabelling and detection system (Amersham Int.). This procedure uses terminal transferase to attach fluorescine-labelled dUTP to the 3' end of the oligonucleotide. The labelling reaction mix were 100 pmol of probe, 5μl fluorescein-11-dUTP, 16μl cacodylate buffer and 16 μl terminal transferase in a total volume of 160 μl. The reaction mix was incubated at 37°C for 90 min. Before hybridization, the reaction mix was checked for effective labelling using the rapid labelling assay following the procedure given in Amersham). An aliquot of the reaction mix (5μl from each labelling reaction) was dotted at the center of the square (in a 1 cm by 1 cm grid) of a Whatman paper. A negative control (5 μl) consisting of a 1 in 16 dilution of the fluorescein-11 dUTP was also included to confirm the washing stage have removed all unincorporated nucleotide. After a min., the Whatman paper was immersed in prewarmed 2 x SSC, 0.1% SDS (w/v) and incubated at 60 ^{0}C with gentle agitation for 20 min. The sheet was then rinsed with water for 30 seconds and immersed in 75% ethanol for a min., transferred to absorbent surface to remove excess liquid and detected using UV light.

2.10. Southern blotting and hybridization:

The gel was depurinated in 0.25 N HCl for 15 min, rinsed twice with

deionized distilled water and then denatured using 0.5 M NaOH for 30 min. The denatured DNA was then transferred on to the Hybond N+ (Amersham Int.) in 10X SSC (1X SSC = 0.15 M Na_3 citrate; 1.5 M NaCl) for 90 min at 5 Hg using a vacuum blotter (Bio Rad, USA). The blot was then crosslinked using a UV crosslinker for 35 second at 150 mJoules (Bio Rad, USA). The blotted membrane was prehybridized with hybridization buffer for 30 min. at 42°C before the addition of labelled single-stranded oligonucleotide bearing a 3'-hydroxyl group. The hybridization buffer consisted of 5X SSC, 0.1%(W/V) hybridization buffer component (supplied from Amersham), 0.02%(W/V) sodium dodecyl sulphate (SDS) and 0.5% (W/V) blocking agent (from Amersham) Hybridization of the labelled probe to the Southern blot was performed at 47°C for 2 to 17 hours. Membranes were then washed sequentially in 5X SSC, 0.1%(W/V) SDS twice for 5 min at room temperature and in 0.1X SSC, 0.1%(W/V) SDS twice for 15 min at 42°C.

2.10.1. Membrane blocking and antibody incubation.

The membrane was rinsed with buffer 1 (0.15 M NaCl, 0.1 M Tris base, pH 7.5) for 1 min., treated with blocking solution (supplied by Amersham Int.) and washed again with buffer 1 for a min. It was then incubated in 1000-fold diluted horseradish peroxidase conjugated anti-fluorescein antibody (anti-fluorescein HRP) in buffer 2 (0.4 M NaCl, 0.1 M Tris base, pH 7.5) containing

0.5% (W/V) bovine serum albumin (BSA) for 30 min. To remove non-specifically bound antibody the membrane was rinsed 4 times with an excess of buffer 2 for 5 min.

2.10.2. Signal generation and detection.

After removing excess wash buffer from the blot, a mixture of equal amounts of detection solution 1 and detection solution 2 (Amersham Int.) which contained peroxide and luminol respectively were added directly to the blot on the side carrying the DNA and incubated for 1 min at room temperature. Then excess detection buffer was removed and the blot was covered in plastic wrap and exposed to autoradiography film (Hyperfilm- ECL, Amersham Int.) for varying lengths of time. The film was then processed in Kodak GBX developer and fixer (Kodak, U.S.A.).

2.11. Specificity testing:

To check whether or not the 16S rRNA primers were specific for *M.leprae*, different species and strains of mycobacteria, related non-mycobacterial species and some commensal organisms were tested. All mycobacteria except *M.leprae*, except were grown in Lowenstein-Jensen media at 37°Cin anaerobic conditions. The Rhodococcus strains were grown in

Species and Strains	Source*	Culture number
Mycobacterium leprae	a	Clinical isolate
Mycobacterium kansassi	a	HB 4962
Mycobacterium gilvum	a	NCTC 10742
Mycobacterium vaccae	a	ATCC 15483
Mycobacterium fortuitum	a	HB 1792
Mycobacterium phlei	a	NCTC 10266
Mycobacterium smegmatis	a	ATCC14470
Mycobacterium nonchromogenicum	a	NCTC10424
Mycobacterium szulgi	a	NCTC 10831
Mycobacterium gastri	a	W 471
Mycobacterium diernhoferi	**a**	**ATCC 19340**
Mycobacterium aurum	a	A+
Mycobacterium tuberculosis (H_{37} RV)	a	H_{37}RV
Mycobacterium tuberculosis (H_{37} Ra)	a	TMC 201
Mycobacterium chitae	a	NCTC 10485
Mycobacterium xenopi	a	S 9
Mycobacterium bovis	a	ATCC 19210
Mycobacterium flavescens	a	NCTC 10271
Mycobacterium duvalie	a	NCTC 358
Mycobacterium thermoresistible	a	NCTC 10409
Mycobacterium gordonae	a	ATCC 14470
Mycobacterium avium	a	S 42
Mycobacterium simiae	a	ATCC 25275
Mycobacterium chelonei	a	NCTC 946
Mycobacterium rhodesiae	a	ATCC 27024
Mycobacterium scrofulaceum	a	HB 1565
Mycobacterium terrae	a	W 45
Mycobacterium intracellularae	a	ATCC 13950
Mycobacterium gadium	a	S 920
Corynebacterium bovis	b	Clinical isolate
Corynebacterium group D2	b	Clinical isolate
Corynebacterium group G1	b	Clinical isolate
Corynebacterium group JK	b	Clinical isolate
Corynebacterium minutissimum	b	Clinical isolate
Corynebacterium xerosis	b	Clinical isolate
Propionibacterium acnes	b	Clinical isolate
Rhodococcus equi	b	Clinical isolate
Staphylococcus aureus	c	Clinical isolate
Streptococcus pneumonicus	c	Clinical isolate

Table 4. Species and strains used in this study.

* a: Dr. Hokan Miorner, AHRI.
b: Dr. James Dick, Johns Hopkins University.
c: Ms. Gunilla Gunlov, ALERT.

Sabouraud dextrose media at 30°C and both Propionibacteria and Corynebacterial strains were grown anaerobically at 37°C on blood and both blood and chocolate agar respectively. All other bacterial strains were grown aerobically on blood agar.

2.12. Detection of M.leprae in tissue specimens and PCR using GAPDH primers:

Fourteen biopsy samples were obtained from skin lesions of leprosy patients and histologically classified according to the Ridley and Jopling scale. The study group included 3 indeterminate, 1 TT/RR, 6 BT, 1 BL/RR, 2 BL and 1 LL biopsies. RNA extraction and cDNA synthesis were performed for each sample following the usual procedure (Ausubel *et al.*, 1994). The concentration of RNA in each sample was measured spectrophotometrically (using Gene Quant, Pharmacia. Biotech, England) after diluting 1 μl of the extracted RNA with 99 μl of RNAse free water. Equal amounts of RNA (1.5 μg) were added to each tube to synthesize cDNA. Of the total 100 μl cDNA, 10 μl was taken from each sample as template DNA and added to the PCR mix. PCR was performed using Glyceraldehyde Phosphate Dehydrogenase (GAPDH) primers. The thermal profile involved an initial denaturation temperature (94°C) for 3 min., 30 cycles of heat denaturation at 94°C for one min, primer annealing at 60°C for 2 min and primer extension at 72°C for one min. After the 30th cycle, extension was

continued for 5 min. at 72°C. After visualizing the result on gel, the amount of each cDNA needed to give approximately equal PCR signals for GAPDH was determined. The amount of cDNA added (in μl) for the fourteen patient samples starting from lane 1-14 was 2, 5, 4, 5, 12, 14, 3, 5, 5, 4, 0.5, 5, 5 and 1 respectively and that amount was used in subsequent experiments. To see whether or not there were crossreactions, 13 control biopsies (9 patient samples with other skin diseases and 4 normal individuals) were tested in the same manner as for leprosy patients.

2.13. Quantitation of *M.leprae* in patient biopsies:

A known amount of bacteria was serially diluted and subjected to RNA extraction and RT-PCR in parallel with RNA obtained from tissue samples. The signal generated from the biopsy specimen (number of bacteria not known) on the Southern Blot was then compared to PCR signals generated by serially diluted samples containing known numbers of bacteria. Results were expressed as the number of bacteria in equal amounts of tissue after adjustment based on the relative densities of the GAPDH signals obtained by computer assisted densitometry (Pharmacia, England and Sun Microsystem Inc., U.S.A) to control for the relative amounts of cDNA added.

3. RESULTS

3.1. Comparison of extraction systems:

Successful amplification of *M.leprae* by PCR is dependent on the quality and concentration of either the genomic DNA or RNA extracted from the bacillus. Thus, an efficient extraction method should be used so that the system is very sensitive and capable of extracting nucleic acid from skin biopsies of patient samples having very few AFB. Equal volumes of a bacterial suspension obtained from the skin biopsy of a leprosy patient were subjected to two different extraction systems (freeze/thaw and DNA STAT 60™) by dividing the sample into two equal parts. Lysed bacteria and purified DNA (obtained using the freeze/thaw and DNA STAT 60™ methods respectively) were then subjected to PCR amplification with the same reaction conditions. The primers used in this procedure were the 16S rRNA primers P1 and P3 that generate a 231 bp fragment. As shown in Fig. 3A, a better signal was obtained from samples treated with DNA STAT 60™ (lanes 1 and 2) than from samples treated with the freeze/thaw method (lane 3 and 4). After comparing these two methods and choosing the one with better efficiency, comparison was made with the third type of extraction system (RNA STAT 60™). The result shown in Fig. 3B clearly indicated that extraction using RNA STAT 60™ followed by reverse transcription and PCR (lane 3 and 4) gave a stronger PCR signal than PCR of genomic DNA

isolated with the DNA STAT 60™ extraction system.

3.2. Primers for cDNA synthesis:

In order to optimize the conditions for cDNA synthesis, four different primers, oligo $(dT)_{15}$, 16S rRNA antisense primer (P3), 16S rRNA probe (P5) and random hexamers were tested. DNA synthesis reaction mixes, each containing the same template with a different primer, were set up after DNAse treatment to destroy any contaminating genomic DNA, and PCR reactions were set up using equal volumes of cDNA. As shown in Fig. 4**A**, with the exception of oligo $(dT)_{15}$ (lane 1), all samples (lane 2 to 4) generated signals. Since a clear but non-specific signal (could be a primer-dimer artifact) was seen in the sample containing primer #5 (lane 3), we selected the 16S rRNA 3' primer (P3) and random hexamers for further investigation. Using the same procedure the selected primers were tested individually as well as in combination. The best result was obtained when these primers were used in combination (Fig. 4**B**, lane 3). The primers used for PCR were the same as those used for the comparison of extraction systems (P1 and P3).

3.3. Selection of PCR primers:

Different combinations of sense and anti-sense primers were tested to choose the primers that gave not only a strong signal but specifically amplified

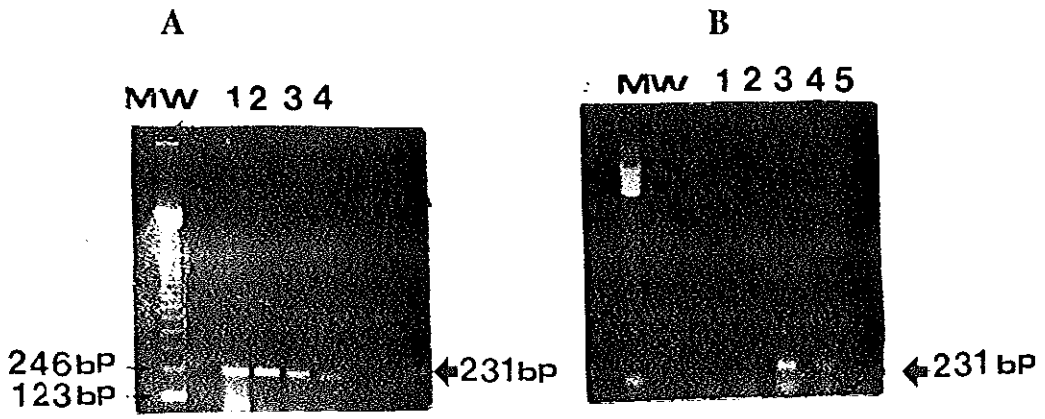

Fig. 3. PCR after extraction of genomic DNA and total cellular RNA. (A) Genomic DNA extracted using DNA STAT 60™ (lane 1 and 2) and cell lysates obtained using the freeze/thaw method (lane 3 and 4). (B) DNA extracted using DNA STAT 60™ (lane 1 and 2), RNA purified using RNA STAT 60™ (lane 3 and 4) and a negative control (lane 5). Lanes 2 and 4 in Fig. **A** and Fig. **B** contain less DNA (10 fold dilution of lanes 1 and 3).

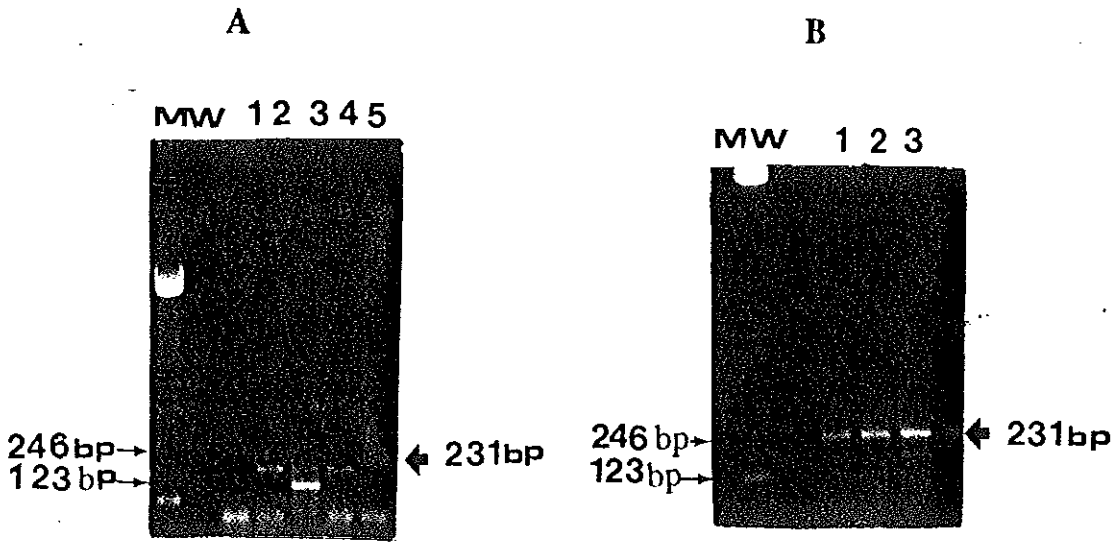

Fig. 4. Comparison of primers for cDNA synthesis. (A) *M.leprae* cDNA synthesis using oligo $(dT)_{15}$ (lane 1), 16S rRNA antisense primer (P3) (lane 2), 16S rRNA probe (P5) (lane 3), random hexamers (lane 4) and negative control (lane 5). (B) *M.leprae* cDNA synthesis using P3 (lane 1), random hexamers (lane 2) and combination of P3 and random hexamers (lane 3). Primers P1 and P3 were used for all PCR reactions.

M.leprae. In preliminary tests cDNAs from *M.leprae* and 28 other mycobacterial species and strains were subjected to PCR amplification using one set of primers (P1 and P3) that generate a 231 base pair product. As shown in fig. 6A first row, detectable amplification was obtained for all mycobacteria. Upon hybridization with labelled probe and non-radioactive detection it was possible to eliminate the signal from 27 different species (data not shown). This was done by increasing the stringency conditions i.e. by increasing the temperature of stringency wash from 42°C to 47°C and decreasing the salt concentration from 1X SSC, 0.1 % (W/V) SDS to 0.1X SSC, 0.1% (W/V) SDS. However, two species, *M.bovis* and *M.avium,* were found to crossreact at all levels of stringency.

Using these two crossreacting species and *M.leprae*, four sets of primers were tested. Primers P2 and P5, which should have generated a 862 bp product, did not give a positive signal. The other 3 sets of primers gave positive signals. However, the primers that generated a 231 bp product (P1 and P3) still crossreacted with other species of mycobacteria. The remaining two sets generating products of 172 bp (P2 and P3) and 405 bp (P2 and P4) gave signals only for *M.leprae,* but a significant difference in sensitivity was observed i.e. the P2 and P3 former gave a stronger signal (Table 5 and Fig. 5). The other two sets of primers (P1 and P4 and P1 and P5 that would generate PCR products of 464 and 921 bp respectively) were not tested.

3.4. Species specific assessment using selected primers.

The objective of this part of the study was to determine which primers specifically amplify *M.leprae* by RT-PCR. Selected oligonucleotides were tested. As a positive control, primers that amplified *M.bovis* and *M.avium* were also tested. When the amplified products were separated in a 1.8% agarose gel and stained with ethidium bromide, a prominent band of amplified DNA was observed for all species of the genus mycobacteria tested using primers P1 and P3, whereas, no signal was obtained for species of Mycobacteria other than M.leprae using primers P2 and P3 (Fig. 6A). Upon Southern blot hybridization of PCR products (Fig. 7), the probe complementary to *M.leprae* hybridized only to the PCR product amplified from *M.leprae* but not to products from any other mycobacterial species investigated. Hence the oligonucleotide pair of primers P2 and P3 appear to be species-specific. They were also tested against a panel of potentially crossreacting related non-mycobacterial species and some nasopharyngeal commensal organisms. All other species failed to generate a positive signal confirming that the primers are species-specific (Fig. 6B). No amplification was obtained when primers P1 and P3 were tested using related non-mycobacterial species as templates (Fig. 6C), confirming the genus-specificity of this set of primers. Bands larger than the intended target fragments, however, were also observed in samples containing *Corynebacterium* species (Fig. 6C, lanes 2, 3, 4, 5 and 7).

3.5. PCR optimization using different buffers, temperatures and adjuncts for two sets of primers.

3.5.1. Genus-specific primers (P1 and P3): To select optimal amplification conditions, twelve different buffers from Stratagene and one buffer from Boehringer Mannheim were compared. Amplification by PCR in buffer 8 and buffer 9 (both from Stratagene) gave stronger signals, the best being buffer 9 (Fig. 8A). Following selection of these buffers, testing of different annealing temperatures (55, 57 and 60°C) was performed. The result shown in Fig. 8B confirmed that an annealing temperature of 60°C (lane 5 and 6) was best for samples containing either of the buffers. Moreover, for all annealing temperatures, the best signal was generated by samples that contained buffer 9. Once the optimal annealing temperature and buffer were selected, PCR amplification was performed using different adjuncts such as BSA, formamide, $(NH_4)_2$ SO_4, glycerol, DMSO and Perfect Match^R DNA Polymerase Enhancer. The strongest signal was obtained using Perfect Match^R DNA Polymerase Enhancer (Fig. 8C, lane 7).

3.5.2. Species-specific primers (P2 and P3): It is preferable to optimize the system when changing primers as optimal conditions vary with primers. Because the above primers lacked species-specificity, we tested the species-specific set of

primers with buffers, adjuncts and different annealing conditions used previously. Fig. 9A shows that buffers 2, 4 and 6 (from Stratagene) and the buffer from Boehringer Mannheim were better than others, but the best signal was consistently generated from a reaction mix that contained buffer 2 (from Stratagene). By using buffer 2 with the different adjuncts (Fig. 9B) and different annealing temperatures (data not shown), we showed that Perfect Match^R DNA Polymerase Enhancer (Fig. 9B, lane 6) and a temperature of 60°C (data not shown) gave the best signals. These optimum conditions were used for all subsequent experiments.

3.6. Assessment of sensitivity using species-specific primers (P2 and P3):

The sensitivity of the primers was ascertained by amplification of the 172 bp fragment from serial dilutions of AFB isolated from a patient skin biopsy and counted microscopically. Serial 10 fold dilutions were made from a suspension of *M.leprae* that contained 2.3 x 10^6 bacteria down to 2.3 bacteria. As shown in Fig. **10 A**, the intensity of the signal decreased proportionally i.e. more diluted samples have a faint signal than less diluted ones. The sample which contained only 23 bacteria showed a faint 170 bp fragment after PCR amplification. No detectable band was observed either on agarose gel or on the more sensitive Southern blot (FIG. 10 B) when an extract was added from a suspension containing less than 23 bacteria.

Primer combination	Product size (bp)	Species specificity	Comment
P1 + P3	231	No	CSS*
P1 + P4	464	Not ascertained	NT**
P1 + P5	921	Not ascertained	NT**
P2 + P3	172	Yes	CSS*
P2 + P4	405	Not ascertained	Weak signal
P2 + P5	862	Not ascertained	No signal

Table 5 . Selection of species-specific primers.
Key: * - Consistently strong signal
** - Not tested.

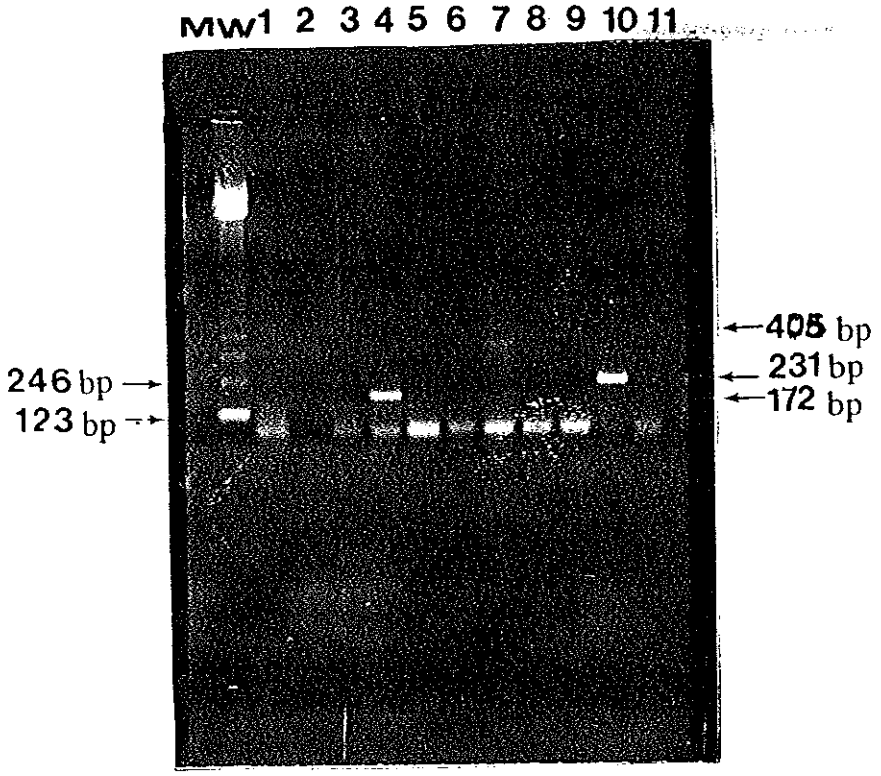

Fig. 5. PCR amplification of *M.leprae* (lanes 1,4 and 7), *M.bovis* (lanes 2, 5, 8 and 10) and M.avium (lanes 3, 6, 9 and 11) using different sets of primers. P2 and P5 (lanes 1-3); P2 and P3 (lanes 4-6); P2 and P4 (lanes 7-10) and P1 and P3 (lane 10 & 11).

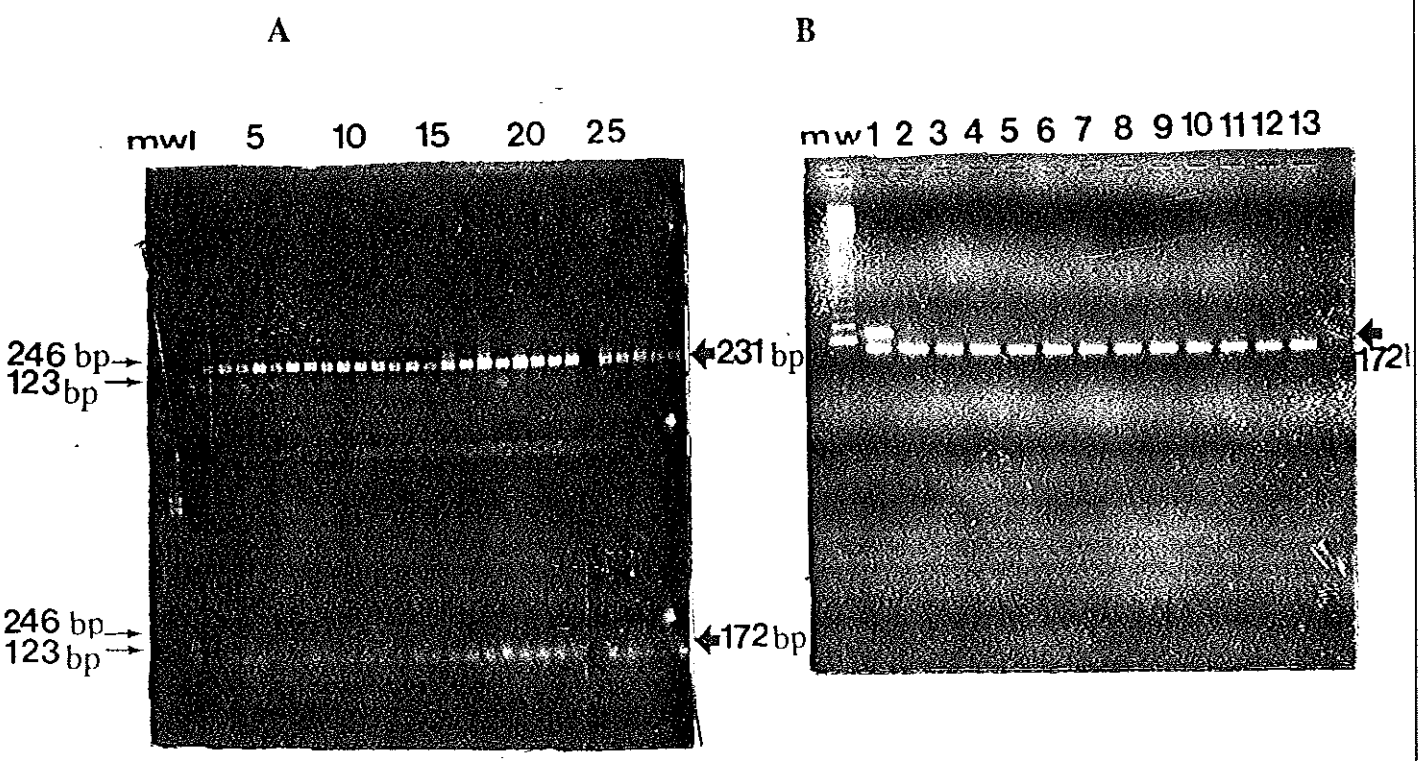

Fig. 6. Specificity of PCR performed using two sets of primers. (A) The first row shows isolates tested with primers P1 and P3 and the second row shows similar strains and species as above primed with P2 and P3. Lane 1 shows *M.leprae* and lanes 2-29 show *Mycobacterium kansassi, Mycobacterium gilvum, Mycobacterium vaccae, Mycobacterium fortuitum, Mycobacterium phlei, Mycobacterium smegmatis, Mycobacterium nonchromogenicum, Mycobacterium szulgi, Mycobacterium gastri, Mycobacterium diernhoferi, Mycobacterium aurum, Mycobacterium tuberculosis ($H_{37}RV$), Mycobacterium tuberculosis ($H_{37}Ra$), Mycobacterium chitae, Mycobacterium xenopi, Mycobacterium bovis, Mycobacterium flavescens, Mycobacterium duvalie, Mycobacterium thermoresistible, Mycobacterium gordonae, Mycobacterium avium, Mycobacterium simiae, Mycobacterium chelonei Ab, Mycobacterium rhodesiae, Mycobacterium scrofulaceum, Mycobacterium terrae, Mycobacterium intracellularae, Mycobacterium gadium.* (B) Lane 1 shows *M.leprae*, lanes 2-12 show *Corynebacterium bovis, Corynebacterium bovis, Corynebacterium group D2, Corynebacterium group G1, Corynebacterium group JK, Corynebacterium minutissium, Corynebacterium xerosis, Propionibacterium acne, Rhodococcus equi, Staphylococcus aureus, Streptococcus pneumonicus* and lane 13 is a negative control, tested with primers P2 and P3.

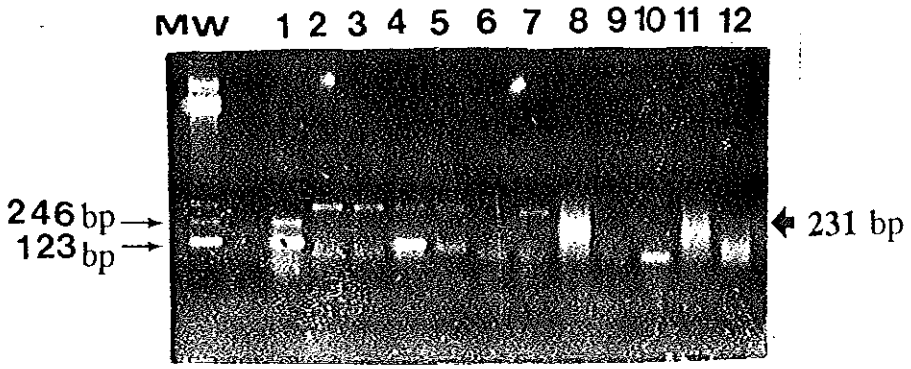

Fig. 6C. PCR amplification using primers P1 and P3. Lane 1 shows amplification of *M.leprae* and lanes 2-12 show amplification of *Corynebacterium bovis (BO-4), Corynebacterium group 1-a, Corynebacterium group D-2, Corynebacterium JK, Corynebacterium minutissium, Corynebacterium xerosis, Rhodococcus equi, Propionibacterium acnes, Streptococcus pneumonicus, Staphylococcus aureus*, and a negative control respectively.

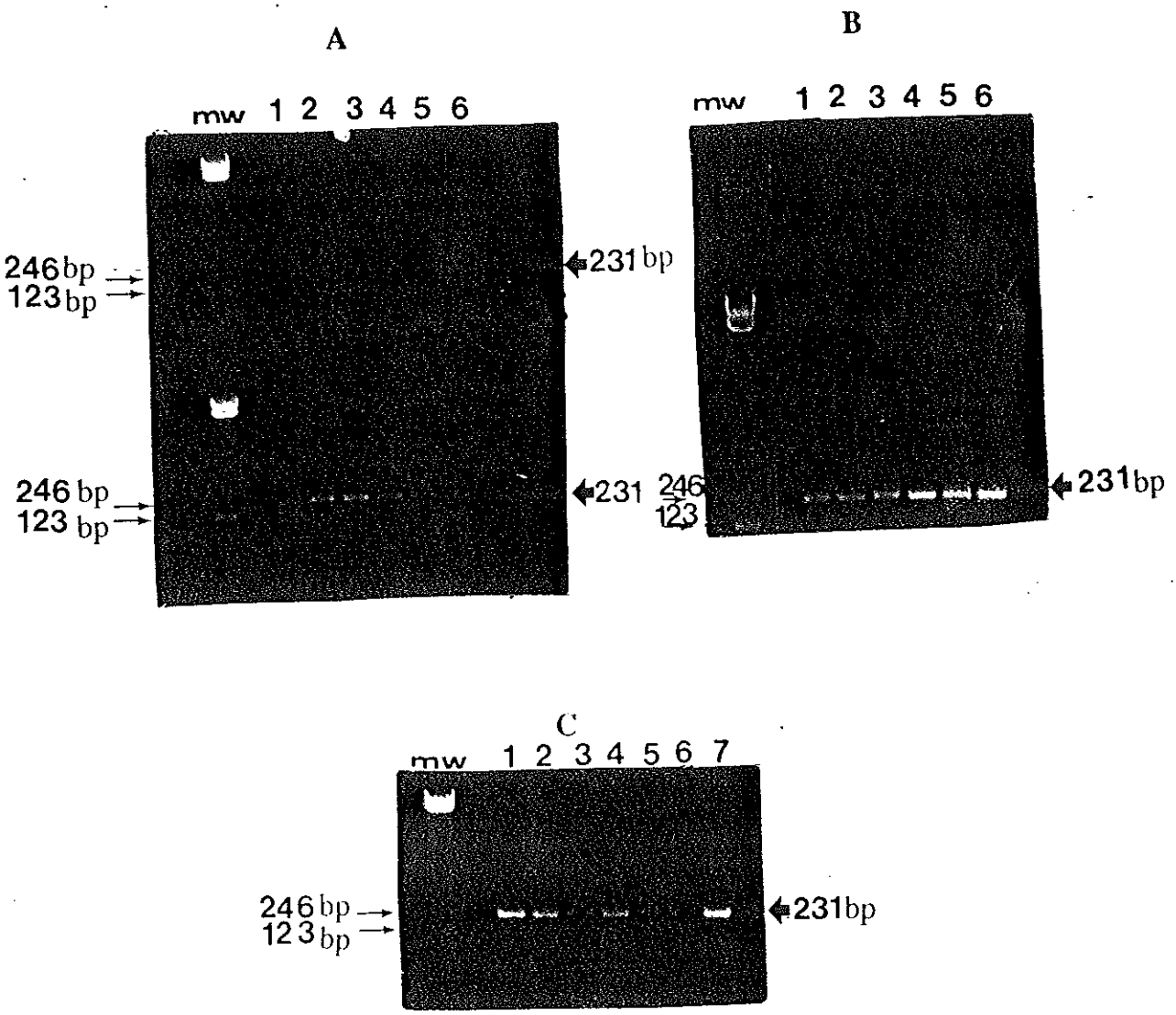

Fig. 8. Optimization of PCR for Genus-specific primers (P1 and P3). (A). First row lanes 1-6 show buffers #1-6 (Stratagene) respectively and second row lanes 1-6 show buffers #7-12 (Stratagene) respectively. **(B).** Buffer #8 (lanes 1, 3 and 5) and buffer #9 (lanes 2, 4 and 6) tested at annealing temperatures of 55°C (lane 1 and 2), 57°C (lane 3 and 4) and 60°C (lane 5 and 6). **(C).** Samples treated with different adjunets: A control with no adjunct (lane 1) Formamide (lane 2), BSA (lane 3), DMSO (lane 4), glycerol (lane 5), $(NH_4)_2 SO_4$ (lane 6) and Perfect Match DNA Polymerase Enhancer (lane 7).

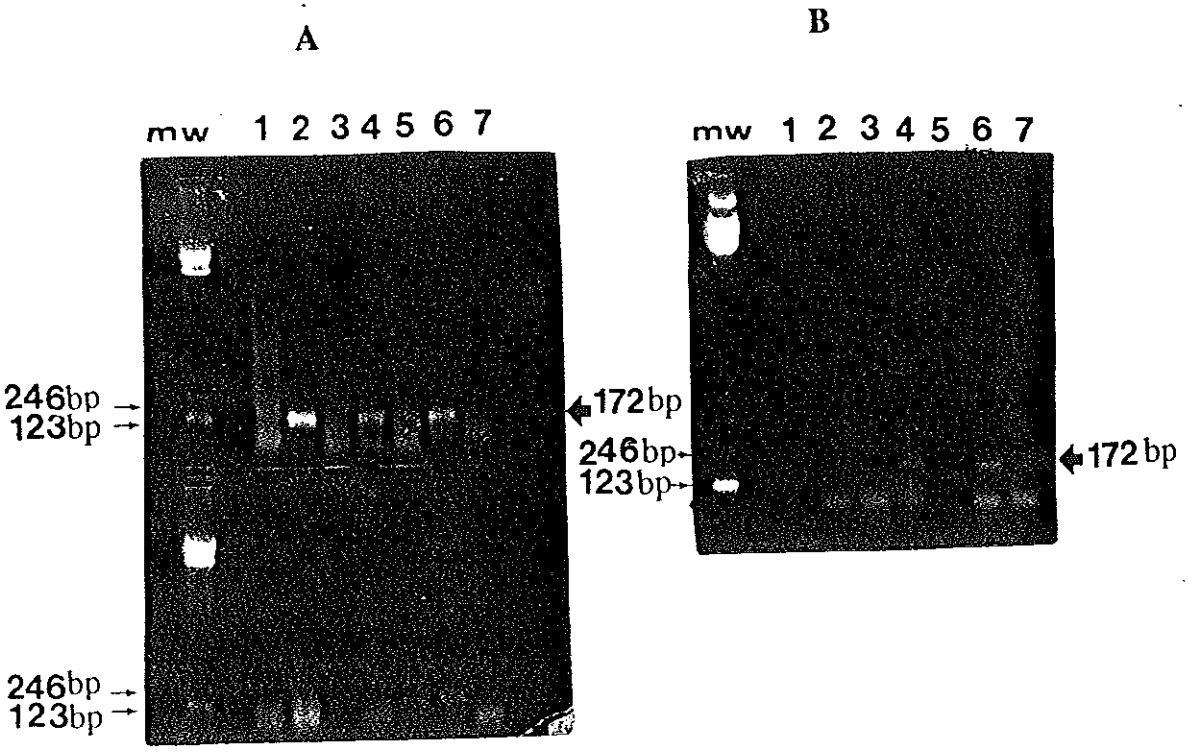

Fig. 9. Optimization of PCR for species-specific primers (P2/P3). (A). First row lanes 1-7 show buffers #1-7 (Stratagene) respectively; second row lanes 1-5 show buffers 8-12 (Stratagene) respectively; lane 6 shows the buffer from Boehringer Mannheim and lane 7 is a negative control. **(B).** Samples treated with different adjuncts: Formamide (lane 1), BSA (lane 2), DMSO (lane 3), glycerol (lane 4), $(NH_4)_2 SO_4$ (lane 5) and Perfect Match DNA Polymerase Enhancer (lane 6) and a negative control (lane 7).

3.7. RT-PCR detection of *M.leprae* RNA in clinical samples from leprosy patients.

The usefulness of RT-PCR for detection of *M.leprae* in clinical samples was determined by analyzing fourteen biopsy samples from leprosy patients with clinical diagnoses ranging across the leprosy spectrum. RNA extraction, cDNA synthesis and normalization of input cDNA after amplification of GAPDH (Table 6 and Fig. 11**A**, first row) were done. A standard curve to insure linearity of the PCR was also done using GAPDH primers (Fig. 11**B**, second row) by serially diluting the sample 2 fold. PCR for *M.leprae* was performed by adding the same amount of cDNA used for GAPDH primers. RNA obtained from biopsy specimens of all MB patients with BIs ranging from 3 to 6 (lanes 3, 11, 12, 13, and 14) and 8 PB patients (BI ranging from 0 to 1) generated signals after cDNA synthesis and PCR for *M.leprae* (Fig. 11**A** first row). RNA extracted from one sample of a PB leprosy patient (TT/RR, BI=I), however, did not generate any amplified product (Fig. 11**A**).

3.8. Detection of *M.leprae* RNA in clinical samples from patients with other skin diseases and normal individuals by PCR.

To ascertain the rate of false positives, 13 biopsy samples (9 from patients with skin diseases other than *M.leprae* and 4 from normal individuals) were

tested using GAPDH primers (Fig. 13A and Fig. 13C lanes 1-4) and species-specific P2 and P3 primers (Fig. 13A and Fig. 13C lanes 6-9). Although all samples treated with GAPDH primers generated signals, no amplified products were seen from any of the samples amplified with P2 and P3.

3.9. Quantitation of *M.leprae* in clinical specimens by densitometry.

Quantitation of *M.leprae* from the fourteen patient samples across the leprosy spectra was performed. A standard curve for the number of *M.leprae* was made by serially (10 fold) diluting the sample which contained known number of bacteria (Fig. 14) and using species-specific primers to amplify *M.leprae*. The number of bacteria in patient samples was determined by comparing the intensity of signals generated from patient biopsies to signals generated for serially diluted samples using the standard curve (Fig. 14). The number of bacteria ranged from 0 to 6,738,269 organisms (Table 7). The number of bacteria corresponding to the density of the sample which was not amplified was subtracted for each biopsy. Biopsy numbers appear in the same order as seen in figures 11 and 12. The number of bacteria did not correlate well ($r = 0.17$) with the BI (Fig. 15).

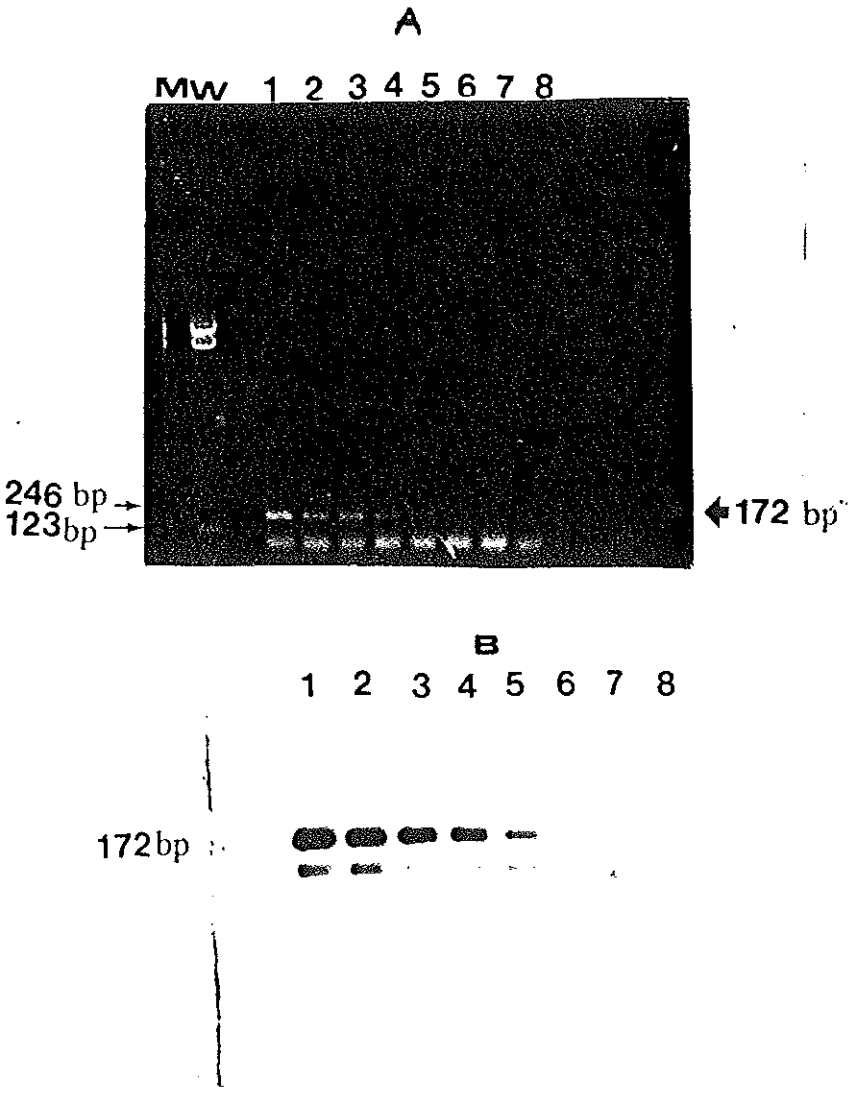

Fig. 10. Amplified PCR product of serially diluted *M.leprae* samples. A. Result obtained from gel electrophoresis. The number of bacteria in lanes 1-7 is 2.3×10^6, 2.3×10^5, 2.3×10^4, 2.3×10^3, 2.3×10^2, 23 and 2.3 respectively and lane 8 is a negative control. B. Southern blot of serially diluted samples. The number of bacteria in lanes 1-7 is 2.3×10^6, 2.3×10^5, 2.3×10^4, 2.3×10^3, 2.3×10^2, 23 and 2.3 respectively and lane 8 is a negative control.

Histological diagnosis	No of cases	No of PCR positive	BI range
Indeterminate	3	3	0-3
TT/RR	1	0	1
BT	6	6	0-1
BL/RR	1	1	4
BL	2	2	5
LL	1	1	6

Table 6. Efficiency of PCR for *M.leprae* detection in patient biopsies.

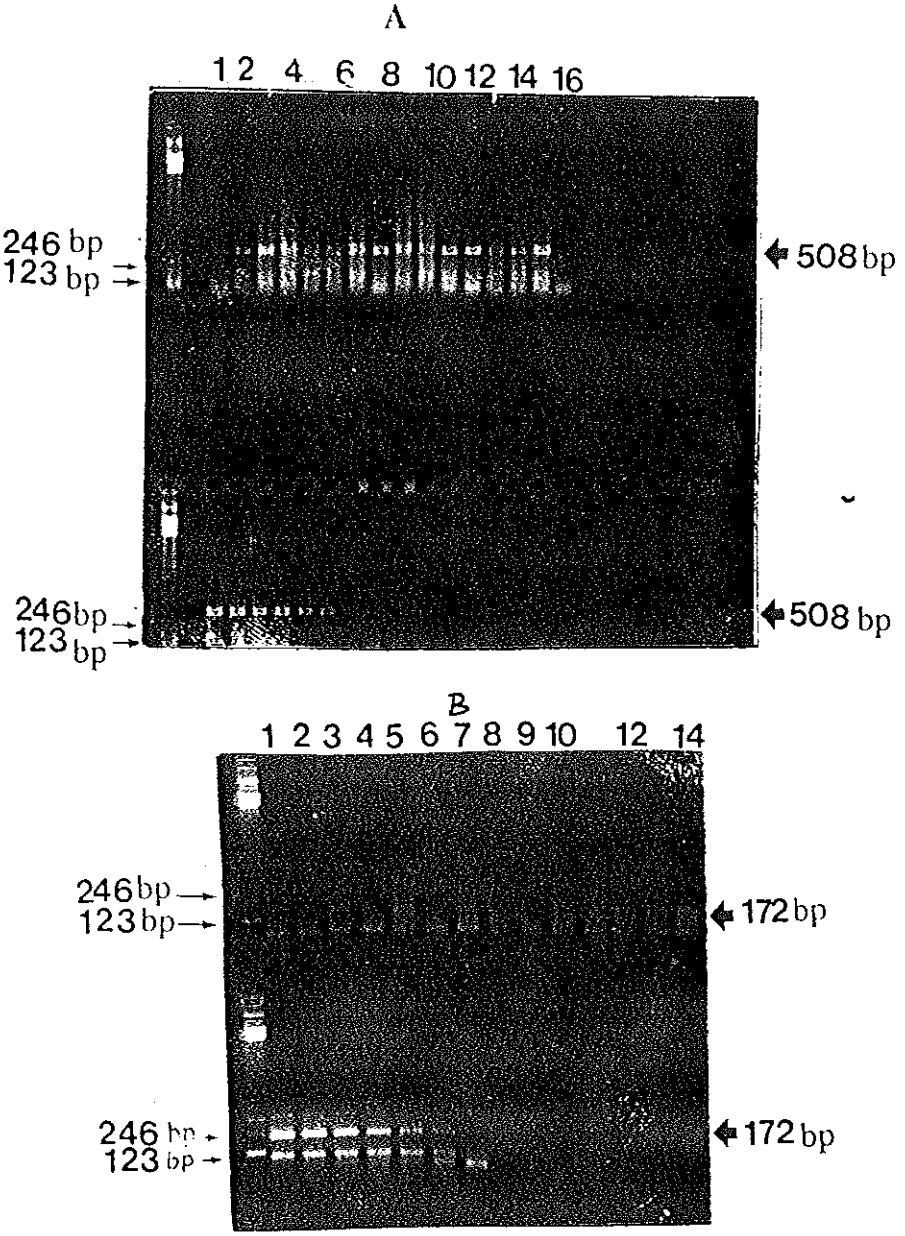

Fig. 11. Gel electrophoresis of *M.leprae* RT-PCR products from biopsies of leprosy patients. A. First row shows a positive control (lane 1), Indeterminate (lanes 2-4), BT (lanes 5, 6, 7, 8, 10 11), TT/RR (lane 9), BL/RR (lane 12), BL (lanes 13 & 14), LL (lane 15) biopsies and negative control (lane 16) amplified using GAPDH primers. Second row shows serial dilutions of *M.leprae* cDNA amplified with GAPDH primers. **B.** First row shows patient samples amplified with 16S rRNA primers (P2 and P3). The order of patient samples is the same as in fig. 11A, but there is no positive control so each is shifted to the left by one lane. Second row shows serial dilution of *M.leprae* (same as in Fig.10).

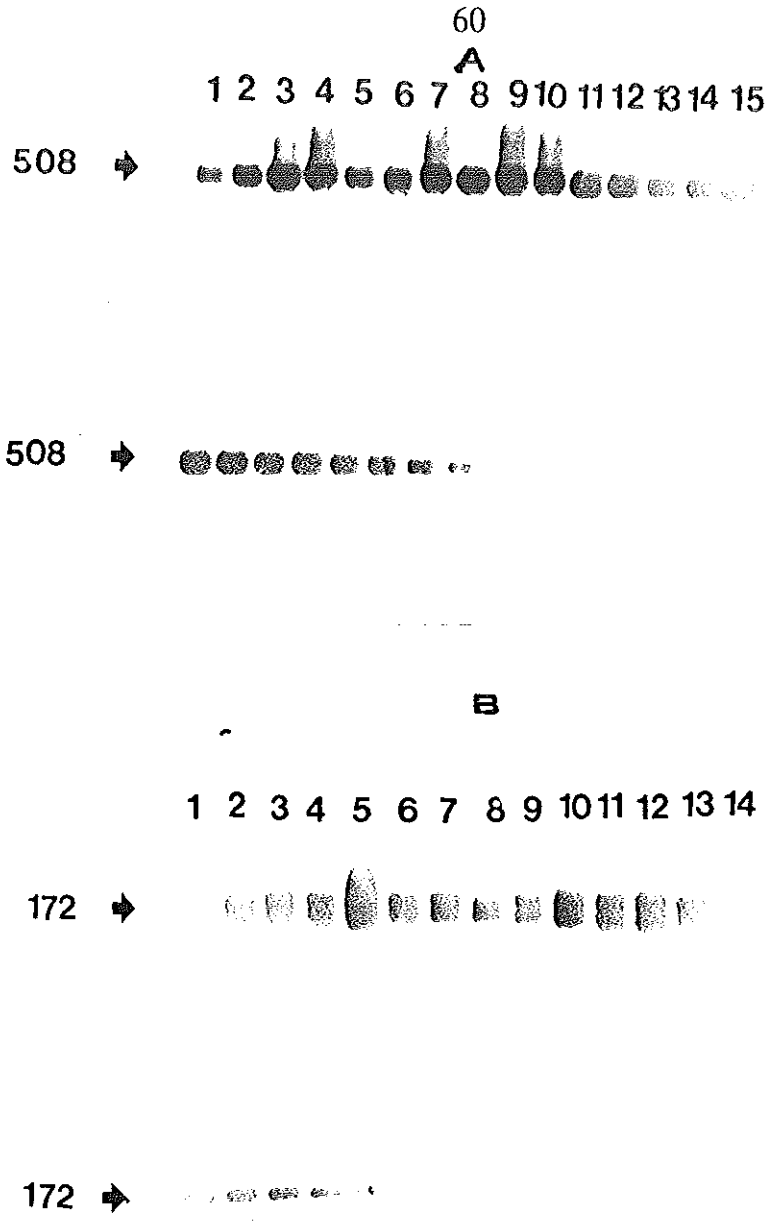

Fig. 12. Southern blot of biopsy samples of *M.leprae* patients with different diagnoses. **A.** First row shows a positive control (lane 1), Indeterminate (lanes 2-4), BT (lanes 5, 6, 7, 8, 10 and 11), TT/RR (lane 9), BL/RR (lane 12), BL (lanes 13 & 14), LL (lane 15) biopsies and negative control (lane 16) amplified using GAPDH primers. Second row shows serial dilutions of *M.leprae* cDNA using GAPDH primers. **B.** shows patient samples amplified with 16S rRNA primers (P2 and P3). The order of patient samples is the same as in fig. 11A, but there is no positive control so each is shifted to the left by one lane.

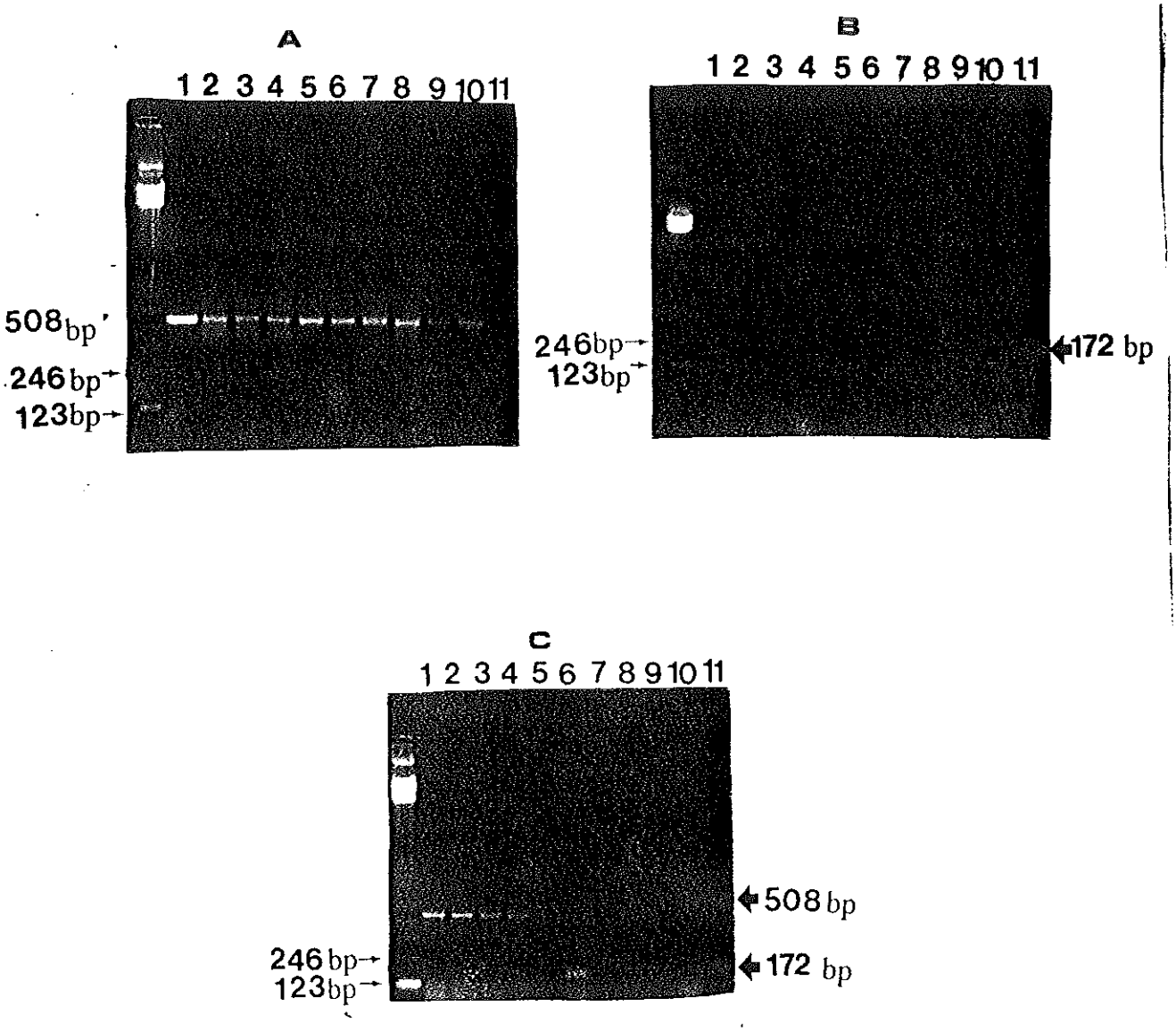

Fig. 13. Amplified PCR products of biopsy samples from patients with skin diseases and normal individuals. A. Patient samples tested using GAPDH primers. Lane 1 is GAPDH positive control (18 hrs PHA stimulated PBMC), lanes 2-10 show patient samples with skin diseases and lane 11 is a negative control. B. Patient samples tested using species-specific primers. The order and number of samples are the same as A. C. Biopsy samples of normal individuals tested using GAPDH primers (lanes 1-4) and species-specific primers (lanes 7-10). Lanes 6 and 11 are positive and negative controls respectively.

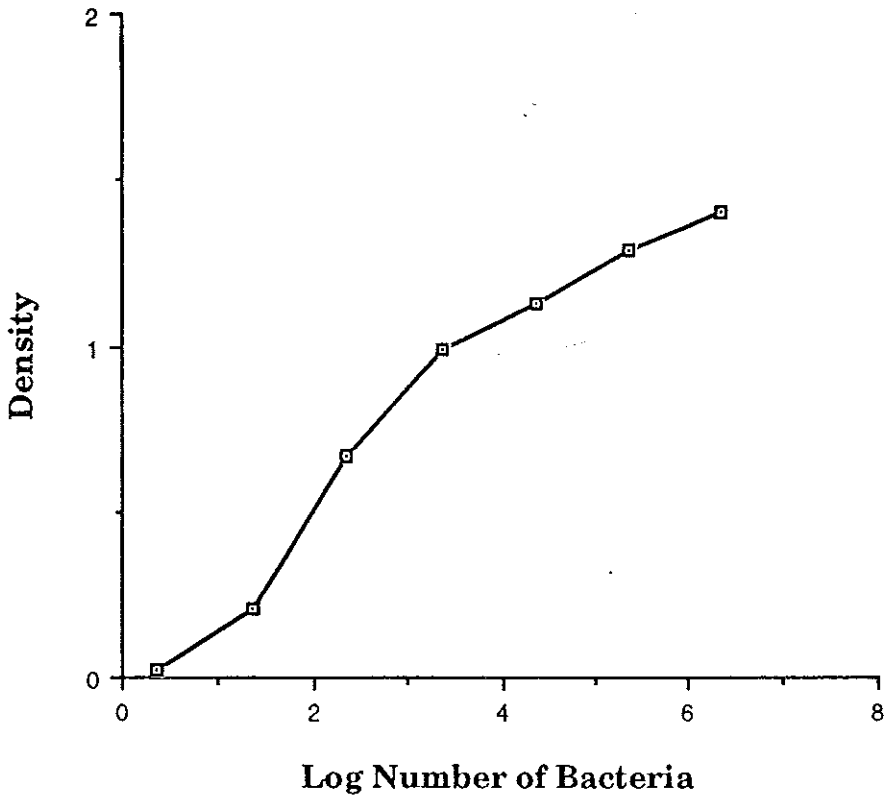

Fig. 14. Density of PCR products in relation to the log number of bacteria.

Biopsy number	BI	Number of bacteria
1	0	124245
2	0	890222
3	3	1017089
4	1	1007278
5	1	6738269
6	1	346088
7	1	827766
8	1	0
9	0	47800
10	1	821313
11	4	599523
12	5	820286
13	5	401137
14	6	342117

Table 7. Quantitation of bacteria for biopsies of leprosy patients using computer assisted densitometry.

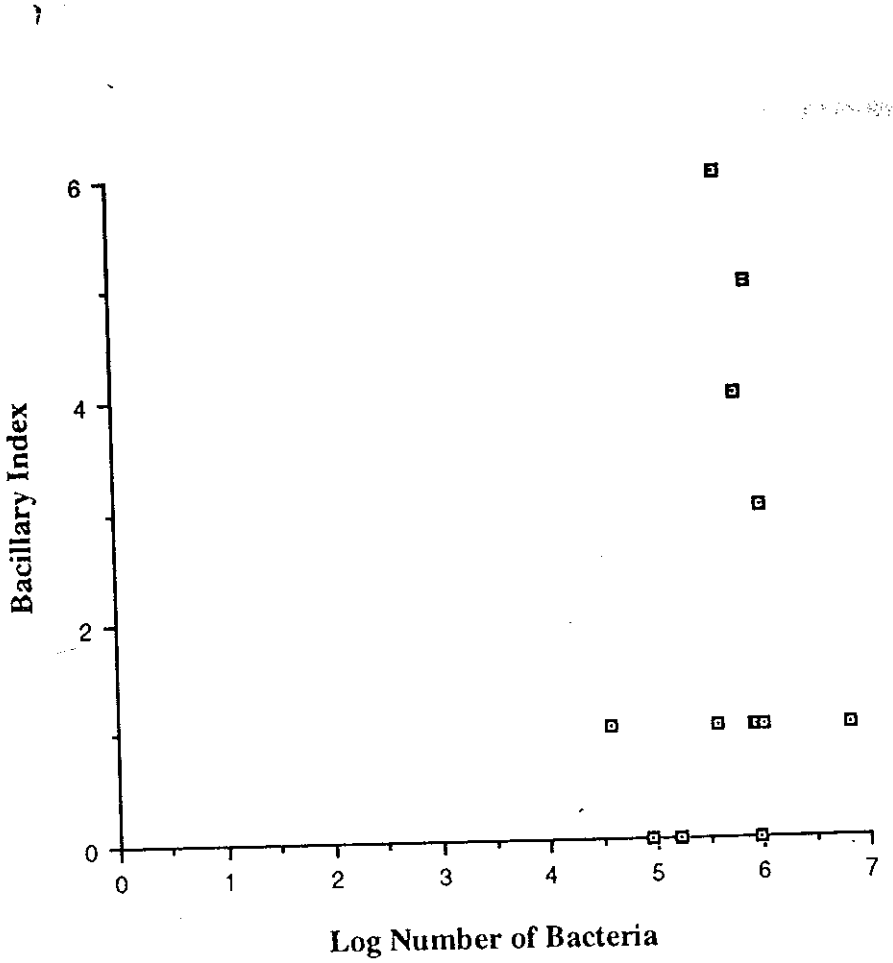

FIG. 15. Correlation between Bacillary Index and log number of bacteria in patient biopsies.

4. DISCUSSION

The efficiency of PCR depends on a reliable extraction system. Due to the presence of a hard protective cell wall, *M.leprae* is refractory to most cell lysis techniques. Since a method that extracts nucleic acids effectively is needed to ensure adequate yields, we tried three different extraction systems. As shown in Fig. 3A, extraction with DNA STAT 60™ was better than using the freeze/thaw technique. However, a better PCR yield was obtained when RNA STAT 60™ was compared with DNA STAT 60™ (Fig. 3B). These observations showed that PCR amplification of nucleic acid purified after chemical lysis and sonication was more efficient than amplification of non-purified nucleic acid obtained by freeze/thawing without subsequent sonication. The best result was obtained when RNA STAT 60™ was used. This was presumably due to extraction of many copies of RNA since there are at least 10^3 to 10^4 copies per cell. Hence, the result we obtained was in agreement with the theoretical expectation.

As no single reaction mixture is optimal for all PCR amplifications, we optimized the system using different buffers following selection of the extraction system. Innis and Gelfand (1990) recommended a PCR buffer containing 10 to 50 mM Tris HCl with a pH range of 8.3-8.8 when measured at 20°C. Determination of optimum $MgCl_2$ concentration is also important because small variations in $MgCl_2$ concentration can cause substantial changes in the PCR yield.

Ausubel *et al.* (1994) suggested three different $MgCl_2$ concentrations (1.5, 3.0 and 4.5 mM) for optimization. It is possible to modify within this range if further improvement of optimization is needed. It is preferable to test as many buffers as possible so as to determine the optimal buffers for the sets of primer used. We tested 13 different buffers and found that Stratagene buffers 9 and 2 gave the strongest signals for genus-specific (P1 and P3) and species-specific (P2 and P3) primers respectively.

Optimization of the system with adjuncts, which enhance PCR in several ways, is also important. BSA and gelatin help to stabilize the enzyme and bind certain PCR inhibitors. Formamide facilitates certain primer-template annealing reactions and is also thought to improve denaturation. DMSO and glycerol have similar effects in that they lower the denaturating temperature of GC-rich DNA. In addition, glycerol helps to stabilize Taq enzyme and hence improve the yield. Perfect Match DNA Polymerase Enhancer is a proprietory adjunct made by Stratagene whcih can substantially improve the specificity as well as the yield of PCR by destabilizing mismatched primer-template complexes and by removing the secondary structures that could hamper normal extension. The adjuncts we tested in the PCR were used as recommended by Stratagene. The strongest signal was obtained for samples that contained Perfect Match DNA Polymerase Enhancer (Fig. 8C, lane 7 and Fig. 9B, lane 7). However, no significant difference could be observed with samples that contained adjuncts other than

Perfect Match DNA Polymerase Enhancer. In some cases (Fig. 8C, lanes 3, 5 and 6) lower signals were observed. This shows that adjuncts might have a negative impact on PCR product particularly if the optimum concentration is not used. For example, the presence of 10% DMSO in the reaction mix impedes the activity of Taq DNA polymerase by about 50% (Innis and Gelfand, 1990).

The temperature and time needed for primer annealing depends on the length and concentration of primers as well as their base compositions. Increasing the temperature not only reduces the chance of incorrect primer annealing but also reduces incorporation of incorrect nucleotides at the 3' end of primer. Hence, by increasing the annealing temperature it is possible to make the system more stringent and thereby increase specificity. This helps to avoid amplification of related species that differ from *M.leprae* by only a few base pairs. In general an annealing temperature which is 5°C below the true melting temperature is considered to be the suitable temperature. The melting temperatures of our primers were 64.2°C and 73.5°C. Taking this into account, three different annealing temperatures were tested. The highest annealing temperature (60°C) gave the best result (Fig. 8B).

The specificity of the *M.leprae* 16S rRNA PCR was assessed for several sets of primers. The 16S rRNA primers (P2 and P3) used in this study were specific for *M.leprae*. (Fig. 6A, second row and Fig. 6B). These results were predicted by the degree of similarity observed when the sequences of the 16S

rRNA gene of *M.leprae*, other mycobacteria, and related non-mycobacterial species were aligned (Fig. 2). There is limited homology between the sequences for primers P2 and P3 among various strains. For example, 10 mismatches (10 of 51 bp) between the primers and the corresponding nucleotides of *Mycobacterium bovis* are present, while 29 mismatches (29 of 51 bp) occur between the primer sequences and the aligned sequence of *Rhodococcus equi*. In the presence of such mismatches, it is not surprising that no signal was observed when these primers were tested using these organisms as templates. Specificity of products was readily detected on gels. Hence, neither detection by Southern hybridization nor any further step is required to confirm species specificity using primers P2 and P3. In contrast, primers P1 and P3 amplified all mycobacterial species and strains tested (Fig. 6A, first row). However, no signal could be obtained when they were tested with related and commensal organisms, confirming that they are genus-specific.

Sometimes non-specific bands with molecular sizes lower than the expected product were observed. Upon Southern hybridization using the 16S rRNA probe and highly stringent conditions, we were able to eliminate them. In addition non-specific bands of larger molecular sizes than the specific product were detected (Fig. 6C). Miyazaki, *et al.* (1993) explained that such phenomenon might occur in the presence of random annealing of PCR products or in the absence of sufficient denaturation time for DNA fragments. Because

non-specific larger bands were rarely seen and our denaturation time was consistent, we think that these bands are unlikely to be due to insufficient denaturation. They may be due to non-specific annealing or more likely, cross-reaction.

The sensitivity of the PCR using specific primers was tested by diluting counted *M.leprae* before extraction. The detection limit was found to be 23 bacteria. The consistency of this result was not determined since we were unable to obtain further patient samples with large numbers of viable bacilli. Samples with a high BI but with very low or zero MI were tested several times. However, satisfactory amplification could not be obtained. We expected that a better sensitivity would have been observed had we tested more samples containing many live bacilli. Nevertheless, this method is much more sensitive than microscopic examination since we were able to generate signals from samples which did not contain sufficient mycobacteria to be detected on direct microscopic examination. The use of 16S rRNA primers for PCR amplification theoretically provides higher sensitivity over other PCR protocols targeting single copy genes. In this study, however, a sensitivity comparable to, but not better than, those techniques which use primers that amplify a single copy of target DNA could be observed from isolated organisms (Hartskeerl et al., 1989).

The potential usefulness of this procedure for clinical diagnosis was investigated using species-specific primers to detect *M.leprae* in biopsy samples

from leprosy patients. Of the 14 biopsy samples analyzed, 13 of them (93% of the samples) were PCR positive. Biopsies from 8 out of 9 (89 %) PB patients and 5 out of 5 (100 %) of MB patients were amplified by PCR. The absence of a positive signal in one patient sample (TT/RR) was probably due to the absence of live AFB. We were able to amplify *M.leprae* 16S rRNA from all samples of PB patients having no detectable AFB by microscopic examination. Yoon *et al.*, (1993) also reported that 70% of biopsy samples with BI=0 showed amplification using *M.leprae*-specific RLEP primers. Hence, it seems evident that PCR is more sensitive than other techniques in detecting *M.leprae* in biopsy specimens with low levels of bacteria (as few as one). Although the number of samples tested in this study was too small to determine conclusively, our method appeared to be more sensitive than other PCR methods in detecting *M.leprae* in clinical samples. Yoon *et al.* (1993) amplified DNA using RLEP primers from 99% (72/73) of biopsies from MB patients but only 79% (23/29) of biopsies from PB patients. Jamil *et al.* (1993) also detected DNA from biopsy samples obtained from all MB patients. However, DNA obtained from biopsies of only 3 out of 9 (33%) PB patients was amplified. From a total of 26 patient samples, Gillis *et al.*, (1993) were able to amplify DNA from only 12 MB biopsies (n= 18) and 3 PB biopsies (n= 8).

The specificity of the P2 and P3 primers was confirmed using additional clinical control samples. No false positive *M.leprae* amplification was observed

when skin tissues from patients with various skin diseases (Fig. 13B) or normal individuals (Fig. 13C) were tested.

PCR amplification using species-specific primers in combination with non-radioactive detection by hybridization with labelled oligonucleotide probes may play a role in the early as well as specific diagnosis of *M.leprae* in clinical specimens. Early diagnosis, along with treatment at an early stage of the disease, might prevent serious nerve damage in leprosy patients. It would not be mandatory to run PCR for all clinical samples since *M. leprae* can be readily detected by microscopic examination of biopsy samples or slit skin smears obtained from MB patients. Therefore, PCR would be more appropriately used for *M.leprae* detection in clinical samples in which no AFB are detectable by microscopic examination. However, more extensive testing of patient biopsies using our M. leprae-specific 16S rRNA PCR protocol should be done to further evaluate its sensitivity.

Amplification of 16S rRNA by PCR may be useful for epidemiological as well as clinical studies in areas where leprosy is widely distributed. In regions where disease prevalence is high, household contacts who acquire the bacteria from infected individuals may constitute a reservoir for transmission of the disease within the population in addition to symptomatic patients. Transmission might be prevented if detection of the bacillus were possible in asymptomatic household carriers as well as in patients. The method we have developed might

be useful for this purpose. However, it should first be adapted for use with specimens obtained by simpler, cheaper and less invasive methods, such as nasal secretions collected using nasal-pharyngeal swabs.

Despite the fact that PCR is a technique which can be applied to microbiologic diagnosis, it has certain drawbacks. It requires a thermal cycler, a reliable supply of electric power, and is too expensive when compared to conventional methods to use as a routine diagnostic technique in developing countries. In addition false positive results may be obtained (Nolte *et al.*, 1993). We utilized separate laboratory areas to set up reactions and detect products, as well as separate pipettes and special tips to prevent aerosolization of samples in order to prevent contamination. Negative controls to detect false positive results included in all PCR runs did not show evidence of contamination. Others (Jamil *et al.*, 1993) have indicated that inhibitors found in some clinical specimens can interfere with the PCR reaction. Our clinical biopsy specimens did not appear to contain PCR inhibitors since GAPDH was amplified from all samples.

Preliminary quantitation by densitometry of numbers of bacteria in each clinical sample was performed (Table 7). Bacterial load did not correlate well with bacillary index (Fig. 15) or diagnosis. There may be several explanations for this discrepancy. First, technical problems with the PCR may be involved. The PCR should be repeated using serial dilutions of cDNA from samples, especially those with strong signals. The original PCR may not have been

performed within the linear portion of the curve relating input cDNA with strength of the PCR signal. Specifically, the signals for samples number 2,3,5,7,10 and 11 are stronger than that for the highest number of bacteria used to generate the standard curve and may fall in the plateau region of the curve. Second, the PCR for the patient samples and the serial dilutions were performed at different times and should be repeated together to control for differences between runs. In addition, the autoradiograph used for densitometry may have been overexposed so that samples with weaker signals would appear to be stronger and thus give erroneously high numbers. From the gel picture (Fig. **11B**, first row), it is clearly seen that the 172 bp band is not well separated from non-specific bands on patient sample so the densitometer is not accurate. Besides, from the same gel picture the patient sample bands appear to correspond with lanes 5/6 of the serial dilutions that is, blot seems to have led over estimation of bacteria. Finally, differences in viability of organisms from different patient samples might be reflected in the PCR results but not the BI's of the samples. The experiment needs to be repeated to distinguish between these possibilities.

Another interesting finding was the absence of a direct correlation between the estimated number of *M.leprae* and the BI. Samples with the same BI showed considerable differences in the number of organisms detected. This may reflect differences in the proportion of live bacilli i.e. samples with similar numbers of

bacteria might differ in the proportion of viable bacteria they contain. Since RNA is prone to early degradation upon cell death, this could be the reason why samples with similar bacterial load showed differences in the intensity of PCR signals. Detection of 16S rRNA might, in fact, be useful for distinguishing live from dead bacilli. Patel *et al.* (1993) determined the viability of *M.leprae* by amplifying the 71 KDa heat-shock protein (hsp) mRNA. They extracted DNA from live and heat-killed bacilli left for 5 hours at room temperature to ensure degradation of mRNA. A 275 bp PCR fragment was amplified only from the RNA extracted from live bacilli. In addition, van der Vliet *et al.* (1994) were able to generate signals from viable *M. smegmatis* but not bacilli exposed to inhibitory or bacteriacidal drugs using the nucleic acid sequence-based technique to amplify 16S rRNA. These findings show that assessment of viability by 16S rRNA amplification is clinically feasible. RNA detection may therefore be useful for assessment of efficacy of chemotherapy as well as for diagnosis.

5. CONCLUSIONS AND RECOMMENDATIONS

We have developed a specific and sensitive RT-PCR method to detect *M. leprae* using 16S rRNA primers. We were able to detect as few as 23 bacteria isolated from clinical samples. We detected *M. leprae* in all biopsies from MB and most biopsies from PB patients, suggesting that the method may serve as a useful diagnostic tool. We were also able to quantitate the number of bacteria from clinical specimens run in parallel with known numbers of serially diluted *M.leprae*. Hence, we recommend that further studies be undertaken to confirm and perhaps improve the sensitivity of the method and to adapt it for use with non-invasive nasal swab specimens. Moreover, the ability of this technique to distinguish live and dead bacilli should be tested to determine whether the technique is useful for difficult diagnostic cases and assessment of response to chemotherapy.

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