Polymerase Chain Reaction for the Detection of *Mycobacterium leprae*

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A polymerase chain reaction (PCR) using heat-stable *Taq* polymerase is described for the specific detection of *Mycobacterium leprae*, the causative agent of leprosy. A set of primers was selected on the basis of the nucleotide sequence of a gene encoding the 36 kDa antigen of *M. leprae*. With this set of primers in the PCR, *M. leprae* could be detected specifically with a detection limit approximating one bacterium. This PCR appears to meet the criteria of specificity and sensitivity required for a useful tool in epidemiology and eventually for the control of leprosy.

INTRODUCTION

Leprosy is still a major health problem in many parts of the world. It is one of the major disabling diseases and imposes a considerable burden in terms of morbidity and social stigma. The lack of methods to identify *Mycobacterium leprae* quickly and specifically has hampered research into the distribution and spread of the bacillus (World Health Organization, 1988). A sensitive and specific method for the detection of *M. leprae* would add an unbiased criterion to the available means of diagnosis and it might allow diagnosis of leprosy at a very early stage before the appearance of clinical signs. Furthermore, studies on leprosy infection could be envisaged which would give information on cardinal issues such as the sources of infection, the number of infected individuals, the clustering of infectious reservoirs, the risk of infection and disease and the effect of prophylactic treatment within a population.

M. leprae cannot be cultured *in vitro*. It can be cultured in modest quantities in animals (e.g. armadillos) after experimental infection, which is expensive and time-consuming (Kirchheimer & Storrs, 1971). Mycobacteria can be visualized by staining followed by microscopy, but this is not specific for *M. leprae*. Current available serological tests show a relatively low sensitivity, which, in combination with the low prevalence of leprosy, limits their application to early case finding and detection of infection (Fine *et al.*, 1988). Even when satisfactory immunodetection is achieved, the problems of detecting colonization and infectivity of carriers before the immune response is initiated will remain. Monoclonal antibodies offer a quick and precise identification of *M. leprae* (Kolk *et al.*, 1985) and DNA probes offer the same, rapid and reliable detection of bacteria (Shoemaker *et al.*, 1985). These methods do not, however, meet the required criterion of sensitivity. Nevertheless, DNA probes offer a route to the more sensitive detection and identification of bacterial DNA, for example of *M. leprae* DNA in clinical samples, through the application of the polymerase chain reaction (PCR) using *M. leprae* specific primers. This method is based on the amplification of characteristic DNA sequences (Saiki *et al.*, 1985). PCR has been shown to be very sensitive and, using appropriate primers, specific and has already been applied successfully to the diagnosis of genetic disorders and to the detection of viruses (Saiki *et al.*, 1985; Kogan *et al.*, 1987; Wong *et al.*, 1987; Ou *et al.*, 1988; Shibata *et al.*, 1988).

Abbreviation: PCR, polymerase chain reaction.

Here we describe the development of a PCR for the specific and sensitive detection of *M. leprae* DNA which may have potential use as a tool in the epidemiology and control of leprosy.

METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. *M. leprae* was isolated from spleen tissue of an experimentally infected armadillo (*Dasypus novemcinctus* Linn.) as recommended by the World Health Organization (1980). Armadillo liver tissue was obtained from animals experimentally infected with different isolates of patient-derived *M. leprae*. Cultivable mycobacteria were grown in Sauton liquid medium, heat-killed (10 min at 80 °C) and harvested by centrifugation. Clinical isolates of other bacteria (see Table 1), grown on blood-agar plates, were kindly provided by Dr R. J. van Ketel (Department of Medical Microbiology, Academic Medical Hospital, Amsterdam, The Netherlands).

Preparation of chromosomal DNA. DNA was isolated by a modification of the method described by Okanishi & Manome (1980) for the isolation of DNA from *Streptomyces*. Bacteria were washed twice with Tris-buffered saline/EDTA (150 mM-NaCl, 100 mM-Tris/HCl, pH 8.0, and 10 mM-EDTA) and resuspended in the same buffer (4 mg wet weight ml⁻¹), containing lytic enzyme L1 (12 mg ml⁻¹; BDH) and lysozyme (2 mg ml⁻¹). After incubation for 18 h at 37 °C, proteinase K and SDS were added to final concentrations of 1 mg ml⁻¹ and 1% (w/v), respectively, and incubation was continued for 2 h at 60 °C. SDS was then added to a final concentration of 3.5% (w/v) and the mixture was incubated for another 2 h at 60 °C.

The DNA solution was extracted twice with phenol and chloroform and precipitated with ethanol (Maniatis *et al.*, 1982). After washing with 70% (v/v) ethanol, the DNA was air-dried and redissolved in 10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA to a concentration of 20 µg ml⁻¹.

DNA from human peripheral blood lymphocytes and from homogenized armadillo liver (2 min at 2000 r.p.m. in 100 mM-Tris/HCl, pH 8.0, 10 mM-EDTA) was prepared in the same way as the bacterial DNA. Concentrations of DNA were determined spectrophotometrically at 260 nm and concentrations were verified by comparing the intensities of bands after electrophoresis of various amounts of DNA on 0.7% (w/v) agarose gels with bands of known quantities of DNA.

Southern blot hybridization. Southern blotting (Southern, 1975) and hybridization were done basically as described by van Eys *et al.* (1988). Denatured DNA was blotted onto Zeta-probe nylon membranes (Bio-Rad) according to the manufacturer's instructions. A 1.0 kb *Eco*RI fragment comprising the gene encoding the 36 kDa antigen of *M. leprae* (hereafter referred to as the 36 K gene) (J. E. R. Thole and others, unpublished results) was used as a DNA probe. The DNA was labelled using a DNA digoxigenin-dUTP labelling and detection kit (Boehringer Mannheim) and added at a concentration of 10 ng (ml hybridization mix)⁻¹. Pre-hybridization and hybridization were done at 60 °C for 1 h and 16 h respectively. Membranes were washed sequentially in 2 × SSC, 0.1 × SSC, 0.1% (w/v) SDS and 0.01 × SSC, 0.1% (w/v) SDS for 30 min each at 60 °C (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) (Maniatis *et al.*, 1982).

Oligonucleotides. We compared the DNA sequences of the genes encoding the 65 kDa antigen of *M. leprae* (Mehra *et al.*, 1986) and *Mycobacterium bovis* BCG/*Mycobacterium tuberculosis* (Thole *et al.*, 1987; Shinnick *et al.*, 1987) (these genes will be subsequently referred to as the 65 K genes). We then selected two homologous regions (for *M. leprae*, bp 1365–1385 and bp 1551–1568, and for *M. bovis* BCG, bp 1734–1754 and bp 1920–1937) separated by a DNA segment of 166 bp on each gene (numbering of bp according to Mehra *et al.*, 1986 and Thole *et al.*, 1987, respectively). Oligonucleotides P1 (CTCAAGGAGCGCAAGCACCG) and P2 (TTGAAGGCGATCTGCTT) are complementary to the + and – strands of these homologous sequences. Oligonucleotides S13 (CTCCACCTGGACCGGCGAT) and S62 (GACTAGCCTGCCAAGTCG) are complementary to the + and – strands of sequences positioned 494 bp apart on the gene encoding the 36 K antigen of *M. leprae* (de Wit & Klatser, 1988; J. E. R. Thole and others, unpublished results). Oligonucleotides were kindly synthesized by H. M. Hodemaekers, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands, on a Gene Assembler (Pharmacia). Oligonucleotides were purified by thin layer chromatography using HPTLC Kieselgel 60F254 plates (Merck, FRG) and an elution buffer consisting of 35% (v/v) NH₄OH, 55% (v/v) propanol and 10% (v/v) H₂O.

PCR. The PCR with the heat-stable DNA-polymerase from *Thermus aquaticus* (*Taq*; Perkin Elmer) was done essentially as recommended by the manufacturer. Briefly, 2.5 units *Taq* enzyme were added to 100 µl of a solution of 50 mM-NaCl, 9 mM-MgCl₂, 10 mM-Tris/HCl, 0.2 mg ml⁻¹ BSA, pH 9.6 containing 1 mM of each of the deoxynucleotides dATP, dCTP, dGTP and dTTP (Pharmacia) and, unless otherwise indicated, 100 ng DNA and 30 ng of each primer. PCRs were performed in a PCR-processor (Bio-med) using 45 cycles as follows: 2 min denaturation at 94 °C, 2 min annealing at 55 °C and 3 min primer-extension at 72 °C. After the 45th cycle the extension reaction was continued for another 12 min at 72 °C. Samples of 20 µl of the reaction mixtures were loaded onto 2% agarose gels containing 0.5 µg ml⁻¹ ethidium bromide for electrophoresis (Maniatis *et al.*, 1982). *Hae*III-digested ϕ X174(RF) DNA (New England Biolabs) was used as a molecular size marker.

Table 1. *Bacterial species and strains*

Strain	Species	Source/origin
8346	Armadillo derived mycobacteria	F. Portaels (Portaels & Pattyn, 1982)
5544	<i>Mycobacterium africanum</i>	RIVM*
8063	<i>Mycobacterium avium</i>	F. Portaels
8316	<i>Mycobacterium bovis</i>	RIVM
515	<i>Mycobacterium chelonae</i>	F. Portaels
–	<i>Mycobacterium duvalii</i>	S. R. Pattyn
ATCC 6841	<i>Mycobacterium fortuitum</i>	ATCC†
ATCC 25220	<i>Mycobacterium gastri</i>	ATCC
8270	<i>Mycobacterium gordonae</i>	RIVM
ATCC 15985	<i>Mycobacterium intracellulare</i>	ATCC
1012	<i>Mycobacterium kansasii</i>	RIVM
Armadillo isolate	<i>Mycobacterium leprae</i>	This laboratory
219	' <i>Mycobacterium lufu</i> '	RIVM
3445	<i>Mycobacterium scrofulaceum</i>	RIVM
ATCC 14468	<i>Mycobacterium smegmatis</i>	ATCC
4514	<i>Mycobacterium tuberculosis</i>	RIVM
H37Rv	<i>Mycobacterium tuberculosis</i>	RIVM
ATCC 25949	<i>Mycobacterium vaccae</i>	ATCC
Clinical isolate	<i>Streptococcus pneumoniae</i>	R. J. van Ketel
Clinical isolate	<i>Escherichia coli</i>	R. J. van Ketel
Clinical isolate	<i>Haemophilus influenzae</i>	R. J. van Ketel
Clinical isolate	<i>Klebsiella pneumoniae</i>	R. J. van Ketel
Clinical isolate	<i>Legionella pneumophila</i>	R. J. van Ketel
Clinical isolate	<i>Pseudomonas aeruginosa</i>	R. J. van Ketel
Clinical isolate	<i>Staphylococcus aureus</i>	R. J. van Ketel

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† American Type Culture Collection.

RESULTS AND DISCUSSION

Selection of primers

Two sets of primers were selected on the basis of established nucleotide sequences of the 36 K and 65 K genes to specifically detect *M. leprae* through amplification of characteristic DNA sequences.

Primers S13 and S62 were derived from the nucleotide sequence of the 36 K gene of *M. leprae* (J. E. R. Thole and others, unpublished results). The 36 kDa antigen contains specific as well as cross-reactive epitopes (de Wit & Klatser, 1988). Consistent with this we found that the gene – probably present in one copy in the genome – hybridized moderately to weakly with DNA preparations from other mycobacteria. It is therefore likely that a large proportion of the gene consists of *M. leprae*-specific nucleotide sequences (Hartskeerl *et al.*, 1988; unpublished observations).

Oligonucleotides P1 and P2 were based on two homologous regions within the 65 K genes of *M. leprae* and *M. bovis* BCG (Mehra *et al.*, 1986; Thole *et al.*, 1987). The 65 kDa antigen is a heat-shock protein present in most, if not all, bacteria as well as in eukaryotic cells (Shinnick *et al.*, 1988; Thole *et al.*, 1988; Thole, 1988). Because of the wide occurrence of the 65 K gene, the use of these primers in the PCR was expected to provide a positive internal control for the reaction.

PCR experiments indeed revealed amplification of target DNA of a large number of species with primers P1 and P2, and the specific amplification of *M. leprae* DNA with primers S13 and S62 (Fig. 1).

Specificity of the PCR

Primers S13 and S62 were tested in the PCR with target DNA purified from 25 different species. Primers P1 and P2 were added as an internal control for the reaction. The use of primers S13 and S62 resulted in the specific amplification of *M. leprae* DNA (Fig. 1*a*). No detectable

Fig. 1. Specificity of PCR. PCR was performed with a combination of primer pairs S13/S62 and P1/P2. PCR was performed as described in Methods, with 150 ng of each of the primers P1 and P2 also added to the reaction mixture. (a) Analysis of amplified DNA by electrophoresis on a 2% (w/v) agarose gel; (b) Southern blot analysis of the agarose gel with a 1 kb *Eco*RI fragment comprising the 36 K gene of *M. leprae* as a probe. PCR was performed with target DNA from: *M. leprae* (lanes 2 and 29), *M. vaccae* (lane 3), *M. tuberculosis* (lane 4), *M. bovis* (lane 5), armadillo derived mycobacteria (lane 6), *M. avium* (lane 7), *M. smegmatis* (lane 8), *M. scrofulaceum* (lane 9), *M. gastri* (lane 10), *M. goodii* (lane 11), *M. kansasii* (lane 12), '*M. lufu*' (lane 13), *M. chelonae* (lane 14), *M. duvalii* (lane 15), *M. fortuitum* (lane 16), *M. intracellulare* (lane 17), *M. africanum* (lane 18), *M. tuberculosis* H37Rv (lane 19), human lymphocytes (lane 20), *P. aeruginosa* (lane 21), *H. influenzae* (lane 22), *E. coli* (lane 23), *Staph. aureus* (lane 24), *K. pneumoniae* (lane 25), *L. pneumophila* (lane 26), *Strep. pneumoniae* (lane 27) and no DNA (negative control, lane 28). Lanes 1 and 16 contained molecular size markers. Numbers at left and right indicate sizes in bp.

amplification occurred with DNA from other species, including closely related mycobacteria. The size of the amplified fragment was 530 bp, which is in agreement with the position of the primers in the 36 K gene. The specificity of the PCR was confirmed by a Southern blot of this gel with the 36 K gene as a DNA probe, on which a band at the 530 bp position was visible only in the samples containing *M. leprae* DNA (Fig. 1b). No bands were visible in samples in which DNA from other sources was used in the PCR. PCR mixtures also contained primers P1 and P2, which resulted in the generation of an additional fragment of about 200 bp in all samples (Fig. 1b). The amplification of this fragment virtually rules out the possibility of unsatisfactory reaction conditions. Hence, the lack of detectable DNA processing with primers S13 and S62 in samples containing DNA from sources other than *M. leprae* was likely due to absence of homology between the primers and the target DNA. The sources of target DNA tested included pathogenic and non-pathogenic mycobacteria and other bacteria, as well as human DNA. Because the PCR with primers S13 and S62 gave no amplification of DNA from a number of bacteria which may be present in human derived samples, nor of human DNA under the conditions described, this method is potentially useful for the detection of *M. leprae* in human samples.

The use of primers P1 and P2 in the PCR resulted in the amplification of a single 200 bp fragment of target DNA from most of the species tested (Fig. 1a). Additional bands on agarose gels were observed in only a few samples (Fig. 1a, lanes 2, 8, 13, 15 and 20). Since these amplified fragments were larger than 200 bp in size, and were not detected when annealing was done at temperatures higher than 55 °C (result not shown), they are probably the result of 'mispriming' outside the selected template (Mullis & Faloona, 1987). The varying intensities of the DNA

Fig. 2. Sensitivity of the PCR. PCR was performed with primer pair S13/S62 as described in Methods. (a) Analysis of amplified DNA by electrophoresis on a 2% (w/v) agarose gel of samples with target DNA isolated from 100 μ l of 10-fold sequential dilutions of a suspension of *M. leprae* containing 2×10^7 bacteria ml^{-1} . The numbers of bacteria per sample were as follows: 2×10^6 (lane 1), 2×10^5 (lane 2), 2×10^4 (lane 3), 2×10^3 (lane 4), 2×10^2 (lane 5), 2×10^1 (lane 6), 2×10^0 (lane 7) and 2×10^{-1} (lane 8). (b) Analysis of amplified DNA by electrophoresis on a 2% (w/v) agarose gel of samples which contained target DNA purified from *M. leprae*. A stock solution was diluted to give following amounts of DNA in the samples: 1 μ g (lane 1), 100 ng (lane 2), 10 ng (lane 3), 1 ng (lane 4), 100 pg (lane 5), 10 pg (lane 6), 1 pg (lane 7), 100 fg (lane 8), 10 fg (lane 9) and 1 fg (lane 10). Numbers at left and right indicate sizes in bp.

bands at 200 bp in the various samples (Fig. 1a) may be due to the presence of one or more mismatches between the primers and the corresponding regions of the target DNAs. Nevertheless, a fragment of approximately the same size was amplified from the DNA of each species tested. P1 and P2 are apparently located in a well-conserved region of the 65 K gene. In our case, primers P1 and P2 served as a useful internal control for the reaction conditions and might be useful as such in the testing of clinical samples. These experiments are now in progress.

Sensitivity of the PCR

The sensitivity of the PCR with primers S13 and S62 was determined by adding to the reaction mixtures DNA extracted from 0.1 ml samples of sequential 10-fold dilutions of a suspension containing 2×10^7 purified leprosy bacilli ml^{-1} . Addition of DNA extracted from a 10^6 -fold dilution of the suspension (theoretically, two bacteria) could be amplified sufficiently, and reproducibly, to give a clearly detectable band in an agarose gel (Fig. 2a, lane 7). No band was detectable either on agarose gels (Fig. 2a, lane 8), nor on more sensitive Southern blots (not shown), when an extract was added from a suspension containing theoretically less than one bacillus. The accuracy of both the microscopic counting of the bacteria in the initial suspension, and the dilution thereof, are limited. Nevertheless, these results suggest that the detection limit of the PCR as performed here approximates 1 to 10 bacilli. A similar detection limit with PCR has been reported by Saiki *et al.* (1988).

In order to investigate further the sensitivity of the PCR, an additional experiment was performed in which various dilutions of purified *M. leprae* DNA were added to the reaction mixtures. Addition of 100 fg chromosomal DNA resulted in a detectable band on agarose gels (Fig. 2b, lane 8), whereas no band could be seen when higher dilutions of DNA were added (Fig. 2b, lanes 9 and 10). Given that the size of the *M. leprae* genome is 2.2×10^9 Da (Clark-Curtiss *et al.*, 1985), 100 fg of DNA corresponds to approximately 20 bacteria.

Thus, similar detection limits were found when DNA extracted from various numbers of *M. leprae* and various dilutions of DNA after purification were used. This indicates that the DNA extraction procedure is very efficient even on low numbers of *M. leprae* and that primers based on a single copy DNA are sufficient to detect only a few bacteria. This implies that the PCR described here is much more sensitive than other methods for the direct detection of *M. leprae*, such as microscopic visualization (10^5 – 10^6 bacteria; Yaeger *et al.*, 1966) and DNA hybridization (10^4 bacteria; Shoemaker *et al.*, 1985; Clark-Curtiss, 1988; Eisenach *et al.*, 1988).

Fig. 3. Detection of *M. leprae* in armadillo liver tissue. PCR was performed with primer pair S13/S62 as described in Methods. Analysis of amplified DNA by electrophoresis on a 2 % (w/v) agarose gel of samples which contained DNA extracted from 100 µg of liver homogenate from uninfected armadillos (lane 3 and 4) and from experimentally-infected armadillos which contained 10^7 (lane 5), 5×10^9 (lane 6) and 2×10^{10} (lane 7) *M. leprae* (g liver tissue⁻¹) as determined by microscopic counting. Controls included 200 ng purified *M. leprae* DNA (lane 2) and no DNA (lane 8). Lane 1 contained molecular size markers. Numbers at left and right indicate sizes in bp.

Detection of M. leprae in armadillo liver tissue

As an initial step to explore the use of the PCR on clinical samples, we have applied the PCR to liver homogenates of experimentally-infected armadillos.

PCR was done with target DNA extracted from 100 µg of armadillo liver homogenates containing 10^{10} , 10^9 , 10^7 and 0 bacilli (the latter from uninfected animals) g⁻¹, respectively, as determined by microscopic counting. As shown in Fig. 3, *M. leprae* could be detected in tissues from all three infected armadillos (lanes 5, 6 and 7). No bands were visible in the samples from the two uninfected armadillos (lanes 3 and 4), indicating that the PCR as performed under these conditions did not result in the amplification of armadillo DNA in the sample. Even in the sample containing DNA from the lightly-infected armadillo (10^7 per g liver), a clear positive PCR signal was generated (Fig. 3, lane 5). This indicates that 1000 bacilli can easily be detected in tissues and that the enzymic lysis used (see Methods) is suitable for the extraction of target DNA from bacilli embedded in armadillo tissue and probably also in human tissue.

The armadillos used were infected with different isolates of *M. leprae*. This implies that the PCR described here is likely to be generally applicable and not restricted to the detection of a single isolate of *M. leprae*.

Conclusions

A method for the direct, specific, quick and reliable detection of *M. leprae* would permit epidemiological evaluation of the distribution of *M. leprae* in a population. In combination with treatment at an early stage of the disease, this strategy might prevent serious damage in patients, and eventually effectively break the transmission cycle. However, currently available methods to detect *M. leprae* lack the required sensitivity. Here we have tested the PCR as a potential tool for the detection of *M. leprae*. Using primers based on a sequence of the 36 K gene, we were able to specifically detect purified *M. leprae* bacilli, as well as *M. leprae* in armadillo tissue.

Considering that the detection limit approached one bacillus, this method may be suitable for the epidemiology of large numbers of people, as well as for clinical studies (diagnosis and follow-up) on individual patients and eventually for the control of leprosy.

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