

MOLECULAR DIAGNOSIS

PCR primers that can detect low levels of *Mycobacterium leprae* DNA

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There are several specific PCR-based methods to detect *Mycobacterium leprae* DNA, but the amplicons are quite large. For example, primers that target the 36-kDa antigen gene and are in common diagnostic use yield a 530-bp product. This may be a disadvantage when examining samples in which the DNA is likely to be damaged and fragmented. Therefore, two sets of *M. leprae*-specific nested primers were designed, based on existing primer pairs which have been shown to be specific for *M. leprae*. Primers that targeted the 18-kDa antigen gene gave an outer product of 136 bp and inner product of 110 bp. The primers based on the RLEP repetitive sequence yielded a 129-bp outer product and 99-bp nested product. With dilutions of a standard *M. leprae* killed whole-cell preparation as the source of DNA, both single-stage and nested PCR were performed after optimisation of the experimental conditions. Compared with the 36-kDa antigen gene primers, the 18-kDa antigen gene outer primers were 100-fold more sensitive and the RLEP outer primers were 1000-fold more sensitive. As an illustration of two possible applications of these new primers, positive results were obtained from three skin slit samples from treated lepromatous leprosy patients and three archaeological samples from human remains showing typical leprosy palaeopathology. It was concluded that these new primers are a useful means of detecting *M. leprae* DNA which is damaged or present at a very low level.

Introduction

Mycobacterium leprae, the causative agent of leprosy, is one of the few known pathogenic bacteria that cannot be cultivated *in vitro*. Diagnosis of the disease is based on clinical examination of the patient, histopathology and the demonstration of acid-fast bacilli in skin-slit smears or biopsies. However, the detection limit based on microscopy is *c.* 10⁴ bacilli/ml [1]. To increase the sensitivity and specificity of the detection of *M. leprae*, several authors have described methods based on PCR [2–9]. These have targeted different parts of the *M. leprae* genome and result in amplicons that are relatively large (320–530 bp) compared with those used routinely for the detection of *M. tuberculosis* (123 bp) [10].

The primers designed by Hartskeerl *et al.* [2] are based on the 36-kDa antigen gene and are still used by many

laboratories. Although the amplicon is 530-bp in size, the authors reported that their method allowed detection of a single bacterium. Williams *et al.* [3] used primers for the *M. leprae* 18-kDa antigen gene that yielded a 360-bp product and could detect as few as 100 *M. leprae* in seeded uninfected human skin biopsy preparations. Plikaytis *et al.* [4] described a nested two-step PCR reaction that amplified a 578-bp outer product and 347-bp inner product from the *M. leprae* *groEL* (65-kDa antigen) gene and could detect as little as 3 fg of DNA, which they equated with the amount found in a single bacillus. Woods and Cole [5] based their PCR on an *M. leprae*-specific repetitive element (RLEP), which was found by Yoon *et al.* [6] to be capable of detecting *M. leprae* DNA in 73% of patients with a bacterial index (BI) of 0. This RLEP-based PCR was developed further by Santos *et al.* [7]. These authors optimised the DNA extraction from clinical samples and, with the inclusion of DNA hybridisation, were able to detect 100 ag (10⁻¹⁶ g) of target DNA, equivalent to about one-tenth of the bacterial genome. The amplicon in this case was 372 bp. A nested two-stage PCR based on the RLEP repetitive sequence was developed by Jamil *et al.* [8] with an outer product of 455 bp and inner product of 320 bp.

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However, all these *M. leprae*-specific PCR methods result in amplicons which are quite large. Treated patients may continue to harbour *M. leprae* DNA, particularly in the case of older patients who received dapsone monotherapy [11]. Even treatment with rifampicin, which kills the organisms rapidly, results in DNA degradation over a period of 2–3 months [12] and it has been suggested that this residual DNA, possibly degraded, may play a role in late reactions [11]. Therefore, it is important that residual DNA can be detected.

DNA degrades rapidly after death and fragments over time [13]. Therefore, archaeological specimens are likely to contain fragmented DNA and *c.* 200 bp has been regarded as the maximum size of amplifiable mammalian DNA from ancient samples. There is increasing interest in the use of PCR to detect the presence of bacterial pathogens in human archaeological material, and both *M. tuberculosis* and *M. leprae* have been demonstrated in specimens that date from ≥ 1000 years [14, 15].

Mycobacterial DNA is likely to be better preserved than that from other organisms, because of the hydrophobic lipid-rich cell wall. *M. leprae* cell walls are even thicker than those of *M. tuberculosis*, so may be anticipated to give greater protection to the microbial DNA within them. Even so, the smaller the DNA target sequence, the greater the likelihood of detecting DNA that has been damaged by antimicrobial therapy or by the passage of time.

The aim of the present study was to design primers, based on those currently in use and known to be *M. leprae*-specific, but with a smaller target sequence than those previously described, which would be suitable for use with treated leprosy patients, samples likely to contain degraded or fragmented DNA, and archaeological specimens.

Materials and methods

Oligonucleotides used as PCR primers

Eight primers were devised, which comprised outer and inner nested pairs based on the *M. leprae*-specific repetitive element (RLEP) and the *M. leprae*-specific 18 kDa antigen gene. The sequences and locations of these primers are listed in Table 1. Primers LP1 and LP2 amplify a 129-bp sequence of the RLEP element, which is reported to be present at 28 copies/cell. The second set of primers, LP3 and LP4, bind internally with some overlap to LP1 and LP2, and amplify a 99-bp product. Primers LP7 and LP8 amplify a 136-bp sequence of the 18-kDa antigen gene which is present as a single copy in the *M. leprae* cell. The inner primers LP5 and LP6 amplify a 110-bp piece of the 136-bp product. The performance of these primers was

Table 1. Sequences of oligonucleotide primers for *M. leprae*

Primer	Residue*	Sequence (5'–3')
LP1	490–509	TGCATGTCATGGCCTTGAGG
LP2	618–599	CACCGATACCAGCGGCAGAA
LP3	505–522	TGAGGTGTGGCGTGGTC
LP4	603–586	CAGAAATGGTGCAAGGGA
LP5	622–642	ATCGACTGTTGTTTGCGCAAC
LP6	731–711	CCAGCAACCGAAATGTTTCGGA
LP7	609–629	TCATAGATGCCTAATCGACTG
LP8	744–726	GGCACATCTGCGGCCAGCA

*For primers LP1–LP4 the primer sequence is found in the *M. leprae* RLEP3 sequence X17153. For primers LP5–LP8 the primer sequence is found in the *M. leprae* 18-kDa antigen gene sequence MSGANT18K.

compared with the 36-kDa antigen gene primers S13 and S62 [2].

Skin-slit samples

Fresh skin-slit samples were obtained from two patients from the Sanatorio San Francisco de Borja in Fontilles, Spain and stored at -20°C until use. The patients had initially been diagnosed as having lepromatous leprosy 20–30 years previously. One patient (B1) had been treated with the recommended WHO regimen for 6 months. The second had received dapsone monotherapy 10 years earlier and returned to the hospital with a reversal reaction. One sample was taken at this time (B2) and another (B3) after the completion of the recommended WHO regimen of rifampicin, clofazimine and dapsone for 6 months and steroids 20–30 mg/day. The samples were supplied by the Pathology Laboratory of the Sanatorio San Francisco de Borja, and stated as having a BI of zero.

Archaeological samples

Six archaeological samples were examined. Samples A1 and A2 were obtained from a putative case of leprosy from a mediaeval burial ground in Suraz, Poland. This was the body of a 40–50-year-old male with characteristic changes in the nose on X-ray and in the leg and toe bones. Samples of this specimen were examined from two areas – around the nasal region (A1) and the metatarsals (A2) [16]. Samples A3 and A4 were from a 10–11th century site at Püspövládány, Hungary, and both samples were from *cavum nasale* with periostitis. Samples A5, a metatarsus ('pencil' form), and A6, a fibula, were from a grave in a 14–15th century site at Ópusztaszer-Monostor, Hungary.

DNA extraction

The method was essentially the second protocol described by Donoghue *et al.* [14]. Briefly, *c.* 25 mg of crushed sample were pre-incubated in proteinase K/EDTA, treated with lysis buffer L6 [17] based on guanidium thiocyanate/EDTA/Triton X-100, mixed on a bead beater and the DNA was captured on to silica.

The silica was washed with washing buffer L2 [17], ethanol 70% and acetone, dried and the DNA was eluted by incubation at 65°C. Preparations were stored at -20°C until used. The silica supernates were also processed. These were chilled on ice and 200 µl of Protein Precipitation Solution (Puregene[®] Flowgen Instruments, Lichfield) were added to each tube. Tubes were vortex mixed for at least 20 s, then centrifuged for 3 min. To precipitate DNA, the supernates were added to 600 µl of isopropanol (-20°C) in clean, sterile tubes, mixed by inversion 50 times and then centrifuged for 3 min. After discarding the supernates, tubes were drained on clean, absorbent paper, then washed once with 600 µl of ethanol 70% (-20°C). Tubes were drained once again and dried with loosened lids in a 56°C heating block for 2 h. DNA was rehydrated in 100 µl of water by placing tubes in a water bath at 65°C for 1 h. Preparations were mixed and centrifuged briefly, divided into small volumes and stored at -20°C until used.

Bacterial cell suspension

The source of *M. leprae* DNA was the reagent known as lepromin, which consists of an autoclaved suspension of bacilli extracted from armadillo tissues, which is used for skin testing in epidemiological studies. This is supplied with an estimated bacterial load of 6×10^9 organisms/ml and was supplied by Dr M. J. Colston (National Institute for Medical Research, London). The suspension was divided into small volumes and stored at -20°C until used. Serial 10-fold dilutions were prepared with sterile water (Sigma-Aldrich) immediately before use.

PCR amplification

Stringent precautions were necessary to avoid cross-contamination. Clean protective clothing was worn with frequent glove changes. A separate room was used for handling PCR product, with a different set of pipettors and protective clothing. Pipettors and surfaces were cleaned with neat household liquid detergent, rinsed with ultrapure water and dried with ethanol before use. Sterile tubes and plugged tips were used.

Pre-aliquoted double-strength PCR mix was purchased from (Advanced Biotechnologies, Epsom). The final composition of the PCR mixture (50 µl) was 75 mmol Tris-HCl (pH 8.8); 20 mmol (NH₄)₂SO₄; 1.5 mmol MgCl₂; Tween 20 0.01% v/v; 200 µmol (each) dATP, dCTP, dGTP and dTTP; and 1.25 units *Taq* DNA polymerase. Bovine serum albumin was added to give a final concentration of 10 mM as this has been shown to improve the yield [18]. The primer pair and DNA preparation (5.0 µl) were added to each pre-aliquoted tube plus sufficient water to bring the volume to 50 µl. A tube with water in place of template was always included as a negative control. Primers were used at final concentrations as follows: LP1, LP2, LP3 and

LP4, 60 nM; LP5 and LP6, 100 nM; LP7 and LP8, 200 nM; S13 and S62, 1.0 µM initially, then 100 nM.

Single-stage PCR to detect M. leprae DNA

Amplification consisted of one cycle of denaturation at 95°C for 5 min, primer annealing at 58°C (RLEP primers) or 55°C for 2 min, and DNA extension at 72°C for 2 min. This was followed by 35–45 cycles of 30 s at 94°C, 30 s at 58°C or 55°C and 1 min at 72°C, followed by 10 min at 72°C.

Nested PCR to detect M. leprae DNA

A two-tube procedure was used. In the first stage, the outer primers (LP1/LP2 or LP7/LP8) were added to the PCR mix. Amplification was performed as follows. After strand separation at 94°C for 4 min there were 25 amplification cycles of 94°C for 40 s, annealing at 55°C or 58°C for 1 min, then strand extension at 72°C for 20 s + 1 s increment per cycle. Finally, there was 1 min at 72°C for further strand extension. New tubes of PCR mix were used in the second stage of nested PCR. The inner primers (LP3/LP4 or LP5/LP6) were transferred into each reaction tube, together with 0.5 µl of product which was used as the DNA template. The total volume was made up to 50 µl with sterile water. This second stage PCR cycle was identical to the first.

Gel electrophoresis

PCR product (8 µl) was added to loading buffer (Sigma) 2.5 µl and electrophoresed in a NuSieve 3:1 agarose 3.0% (w/v) gel (FMC Bioproducts, Flowgen) in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA) at 8.8 V cm for 80 min. Amplified DNA was visualised by ethidium bromide staining plus ultraviolet light and was recorded with a Polaroid camera.

DNA sequencing

PCR product was either sequenced directly or was first separated from other components in the reaction mixture by separation on a gel as above, by electrophoresis with TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA.) The selected band (150–250 mg) was removed from the gel with a sterile scalpel blade into a sterile 0.5-ml tube. DNA was extracted from the gel slices with a MERmaid[™] Spin Kit (10–200 nucleotides) (Anachem, Luton). The purified DNA was then sequenced by MWG-BIOTECH GmbH or by an in-house sequencing service in the UCL Department of Biological Sciences.

Results

Single-stage PCR to detect M. leprae-specific DNA

The new primers and primers S13/S62 were tested against 10-fold dilutions of the lepromin reagent.

Initially, an annealing temperature of 58°C was used. The untreated lepromin reagent gave a good yield of *M. leprae* DNA (Fig. 1). The outer RLEP primers LP1/LP2 gave a strong band of the 129-bp product with a 1×10^4 dilution of lepromin. The inner RLEP primers LP3/LP4 and S13/S62 gave weak bands at this dilution. The outer 18-kDa antigen primers LP7/LP8, which had been used at 100 nM, gave little product under these conditions, although the inner primers LP5/LP6 gave only slightly less product at a 1×10^4 dilution than LP1/LP2. Optimisation experiments showed that best results were obtained with 200 nM LP7 and LP8, and an annealing temperature of 55°C. The lower annealing temperature also improved the yield of the LP5/LP6 product.

When 45 cycles of amplification were used, a positive result was obtained from a 1×10^8 dilution of *M.*



Fig. 1. *M. leprae* PCR after 35 cycles of amplification with different primer pairs and *M. leprae* dilutions from 1×10^1 to 1×10^4 . The annealing temperature was 58°C unless otherwise indicated. Top row, lanes 1–3, primers S13/S62 for 530-bp product from 32-kDa antigen gene, dilutions 10^2 , 10^3 , 10^4 ; 4–7, primers LP1/LP2 for 129-bp product from RLEP sequence, dilutions 10^1 , 10^2 , 10^3 , 10^4 ; 8–11, primers LP3/LP4 for 99-bp product from RLEP sequence, dilutions 10^1 , 10^2 , 10^3 , 10^4 . Bottom row, lanes 1–4, primers LP5/LP6 for 110-bp product from 18-kDa antigen gene, dilutions 10^1 , 10^2 , 10^3 , 10^4 ; 5–6, primers LP5/LP6 for 110-bp product from 18-kDa antigen gene dilutions, 10^3 , 10^4 , with annealing at 55°C; 7–10, primers LP7/LP8 for 136-bp product from 18-kDa antigen gene, dilutions 10^1 , 10^2 , 10^3 , 10^4 with annealing at 55°C; 11, Φ X174 *Hae*III digest molecular markers.

leprae with primers LP1/LP2. The end-point with LP7/LP8 was 10-fold less and for primers S13/S62; 1×10^5 was the highest dilution that gave visible product.

Use of nested PCR to detect M. leprae DNA from clinical and archaeological samples

After nested PCR with the 18-kDa antigen gene primers a band of the expected size (110 bp) was visible from archaeological samples A1, A3 and A4 (Fig. 2). These archaeological samples were also positive with the RLEP primers. One archaeological sample, A3, yielded more product than a 1×10^4 dilution of *M. leprae* DNA. There was no product from samples A2, A5 or A6, a DNA extraction control, nor from the PCR negative control. Sample B1 from a treated lepromatous leprosy patient was positive with both the RLEP and 18-kDa antigen gene primers. The first sample from the patient with a reversal reaction (B2) was negative with the 18-kDa antigen primers, but positive when the RLEP primers were used. Sample B3, obtained immediately after completion of the

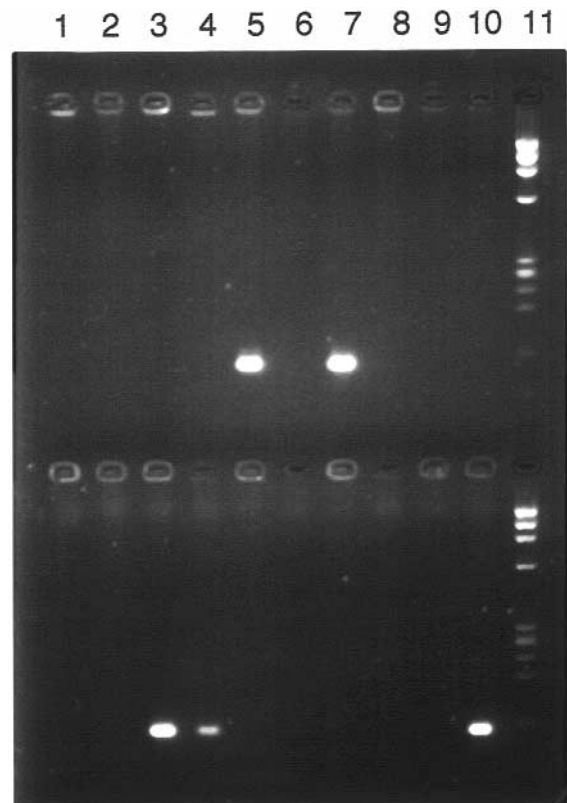


Fig. 2. Nested PCR for *M. leprae* with 18-kDa antigen gene primers. Top row, lane 1, B2 (silica eluate); 2, B2 (silica supernate); 3–4, other negative samples; 5, A1 (eluate); 6, A1 (supernate); 7, A3 (eluate); 8, A3 (supernate); 9–10, other negative samples; 11, Φ X174 *Hae*III digest molecular markers. Bottom row, lanes 1–2, other negative samples; 3 A4 (eluate); 4, A4 (supernate); 5–6, other negative samples; 7, extraction control (eluate); 8, extraction control (supernate); 9, PCR control; 10, *M. leprae* 1×10^4 dilution; 11, Φ X174 *Hae*III digest molecular markers.

WHO treatment regimen, gave a very weak positive result, but only with the RLEP primers.

Use of single-stage PCR to detect M. leprae DNA in clinical and archaeological samples

After 45 cycles of amplification, no positive results were obtained with either RLEP or 18-kDa antigen primers from the treated lepromatous patient samples B1, B2 or B3. However, archaeological samples A1, A3 and A4 yielded products of the expected size with primers LP1/LP2 and LP7/LP8, and appeared to be of equivalent strength to a 1×10^4 dilution of *M. leprae*.

DNA sequencing

With the RLEP outer primers LP1 and LP2, a complete sequence of 129 bases was obtained from archaeological sample A4: TGCATGTCATGGCCTTGAGGTGTC GGCGTGGTCAATGTGGCCGCACCTGAACAGGCA CGTCCCCGTGCACGGTATAACTATTCGCACCTGA TGTTATCCCTTGACCAATTTCTGCCGCTGGTATCG GTG. This identical sequence is found in 30 sequences of *M. leprae* held in the gene data bank. With the exception of an internal 18-bp sequence, a BLAST search of the gene database held by the National Center for Biotechnology Information showed that this product is unique to *M. leprae*.

With the inner RLEP primers LP3 and LP4, a provisional 81-bp sequence was obtained from archaeological sample A3: CCGCACCTGAACAGGCACGTC CCCGTGCACGGTATAACTATTCG-ACCTGATGTTA TCCCTTGACCAATTTCTGNCGCTGG. Where the sequence was obtained from both primers it was identical to that in the database. However, part of the sequence was obtained from only one primer and contained a missing value (-) and one indeterminate result (N).

Preliminary results were also obtained with primer LP8 for the 18-kDa antigen gene and a 108-bp sequence was obtained from the archaeological sample A1: TGTCNGATACTTTAAAGATCCATGGCTAAGACG GGCTTTTGACCGCAGCGGCGTGCTTAGCTTCGTG CTTAGCTTGTTGCGCAAACAACAGTCGATTAGGC ATCTAT. There is one undetermined base (N), but otherwise this sequence has 100% similarity with two sequences of *M. leprae* held in the databank. There is overlap with the primers LP7 and LP8 and the whole length of the intervening DNA has been sequenced.

With the inner primers LP5 and LP6, a 110-bp complete sequence was obtained from the archaeological sample A3 which is identical to the *M. leprae* sequence in the database: ATCGACTGTT GTTTGCGCAACAAGCTAAGCACGAAGCTAAGCA CGCCGCTGCGGTCAAAGCCCGTCTTAGCCATG GATCTTTAAAGTATCCGAACATTTCCGGTTGCTGG. A BLAST nucleotide search revealed that, with the exception of an internal 18-bp sequence, both these

18-kDa antigen gene sequences are unique to *M. leprae*.

Discussion

Two nested pairs of primers were used to successfully amplify *M. leprae* DNA from the lepromin skin test reagent, samples from treated leprosy patients with a BI of zero, and from three of six archaeological specimens dating from the 10th–11th centuries. Nested PCR increases the sensitivity of PCR as it enables a total of 50 amplification cycles to be used, but avoids over-amplification artefacts. It was only with nested PCR based on the RLEP primers that all three clinical samples were found to give positive results. It is not known whether or not residual *M. leprae* DNA is of any clinical significance, so it may be of interest to use these primers, which can detect lower amounts of *M. leprae* DNA than current methods permit, in longitudinal studies of treated leprosy patients.

All three positive archaeological samples were obtained from the *cavum nasale*. It is known that *M. leprae* localises to Schwann cells and nasal epithelial cells [19], and that this can result in an overwhelming rhinitis associated with very high numbers of the organism. The three *cavum nasale* specimens demonstrated the typical palaeopathology associated with leprosy, so it was likely that *M. leprae* DNA was in these samples at the time of death of the host. The detection of *M. leprae* in specimens >1000 years old was described previously in a preliminary report [15] and these additional observations, plus DNA sequencing data, confirm the persistence of *M. leprae* DNA. Leprosy is primarily a disease of peripheral nerves and skin, but affects bones as well. However, the absence of detectable *M. leprae* DNA in the metatarsal specimens (A2, A5) and the fibula sample (A6) need not imply that the remains were from individuals who were not suffering from the disease. Although many skeletons have been found with changes associated with lepromatous leprosy, the peripheral bones are likely to contain relatively little *M. leprae* DNA compared with the nasal region. Furthermore, most observers consider the changes in the limbs to be due to secondary infection, which is the result of loss of sensation due to nerve damage by the organism [20], so some affected limbs may contain no *M. leprae* DNA at all.

All the new primers were of greater sensitivity than the commonly used primers described by Hartskeerl *et al.* [2] and enabled the *M. leprae*-positive archaeological specimens to be detected by single-stage in addition to nested PCR. One reason for this difference is the smaller target size, as this is more likely to result in successful PCR assay of damaged and fragmented DNA. The most sensitive primers were based on the repetitive sequence (RLEP), which is repeated 28 times in the *M. leprae* chromosome. The primers based on

the 18-kDa antigen gene, present as a single copy per cell, were *c.* 10 times less sensitive, whereas the Hartskeerl primers, which result in a 530-bp product, were 1000-fold less sensitive. The lepromin reagent contains *c.* 10⁹ organisms/ml, but it is not known if all the DNA is available for amplification. Therefore, it is calculated that primers LP1/LP2, in a single-stage PCR with 45 amplification cycles, detected at least 0.3 of an organism, and it is very likely that the sensitivity is still greater.

It was concluded that these newly described primers have greater sensitivity in detecting *M. leprae* DNA in samples where the quantity and quality of the residual DNA is likely to be low. Appropriate applications for these primers may be longitudinal studies of treated leprosy patients, and palaeopathological investigations.

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