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Use of PCR-mediated amplification of *Mycobacterium leprae* DNA in different types of clinical samples for the diagnosis of leprosy

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Summary. DNA of *Mycobacterium leprae*, obtained by a highly efficient nucleic acid extraction procedure, was used for standardisation of the amplification of an *M. leprae*-specific repetitive sequence by use of the polymerase chain reaction (PCR). With pure DNA, *M. leprae*-specific amplification was obtained with as low as 100 ag (1 ag = 10^{-18} g) of target DNA, a quantity equal to about one-tenth of the bacterial genome. Optimal processing of different types of clinical samples such as biopsy material, blood and lymph fluid, from multibacillary leprosy patients, was studied. Simple freezing-boiling cycles in the presence of Triton X100, with some additional sample-specific modifications such as pre-treatment with NaOH to eliminate PCR inhibitors, was found to be sufficient to yield amplification of bacterial DNA in samples from paucibacillary patients. Clinical samples from 27 untreated leprosy patients, covering the various clinical forms of the disease, and with a bacterial index ranging from 5+ to 0, were collected and processed for PCR analysis. After hybridisation of the amplified material with a specific sequence, 25 of 27 patients analysed gave positive results for *M. leprae* in at least one of the samples. The potential of PCR for the diagnosis of leprosy is discussed.

Introduction

Identification of *Mycobacterium leprae* is difficult, partly due to the inability of the leprosy bacillus to grow *in vitro*. The diagnosis of leprosy is based on microscopic detection of acid-fast bacilli in tissue smears, in combination with histopathological and clinical evaluation. Recently, the use of various serological and biochemical methods or the use of nucleic acid probes has been suggested for diagnosis.[1] However, none of these has shown sufficient sensitivity or specificity to serve as a diagnostic tool for leprosy.

In the last few years, several reports on the use of the polymerase chain reaction (PCR) for detection of *M. leprae* have been published.[2–7] The strength of PCR is its extreme sensitivity, and, with careful choice of primers, high specificity. For specific amplification of *M. leprae* DNA, the following systems were developed. Woods *et al.*[2] detected small numbers (10^2) of bacteria by amplifying an *M. leprae*-specific repetitive sequence. A similar number of bacilli could be detected by Williams *et al.*,[5] by amplification of a large part of the gene coding for the 18-kDa antigen. Hartskeerl *et al.*[3] were able to detect DNA from a few bacteria by amplification of part of the coding sequence of the 36-kDa antigen. Plikaytis *et al.*[4] detected as little as 3 fg of *M. leprae* genomic DNA by use of nested primer PCR of a portion of the *groEL* gene.

PCR has already been used successfully for the detection of a large number of micro-organisms present in diverse tissues,[8] including the diagnosis of tuberculosis, a mycobacterial disease caused by *M. tuberculosis*, by applying PCR to different types of clinical samples.[9] Although the specificity and sensitivity of most systems used for detection of *M. leprae* is high, thus far data have been presented only on the application of PCR to human skin samples reconstituted with *M. leprae*,[2,4,5] or to biopsy samples from leprosy patients.[7,10] As biopsy sampling is invasive for the patient and skin lesions cannot always be found, we investigated whether clinical samples other than biopsy material could be used for the diagnosis of leprosy. For this purpose, we used PCR amplification of an *M. leprae*-specific repetitive sequence as described by Woods and Cole.[2]

Materials and methods

Bacterial strains and culture conditions

M. tuberculosis strains $H_{37}Rv$ and $H_{37}Ra$ (a gift from Dr A. Werneck, Tuberculosis Reference Center Prof. Helio Fraga, RJ, Brazil) and *M. bovis*-BCG

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(kindly provided by the Ataulfo de Paiva Foundation, RJ, Brazil) were grown in Middlebrook 7H9 broth (Difco) at 37°C for 21 days. *M. smegmatis* ATCC 19420, *M. avium* ATCC 25291 and *M. kansasii* ATCC 12478 were grown in Roux bottles containing 400 ml of Sauton medium at 37°C for 17–20 days. *M. szulgai* NCTC 10831 was grown in the same conditions for 27 days. After growth, bacterial suspensions were centrifuged and the pellet was stored at −20°C until required. All species were also grown on Lowenstein-Jensen medium (Difco) and stored at 4°C. *M. leprae* was purified from infected armadillo tissue as described previously.[11]

Preparation of DNA

Mycobacterial DNA was prepared and purified as described previously.[12] Briefly, cells were mixed with glass beads, phenol and TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7·0) and disrupted by vortex mixing for 3 min. The suspension was submitted to chemical lysis with sodium deoxycholate, followed by deproteinisation of nucleic acids with phenol:chloroform (1:1). *M. leprae* DNA was prepared in the laboratory of Dr M. J. Colston (National Institute of Medical Research, London), by a slightly modified procedure for large scale DNA preparation.[12] In summary, frozen bacilli were disrupted in a cooled mortar with glass beads, followed by the addition of sodium deoxycholate and potassium perchlorate. Subsequent chloroform extraction and DNA precipitation yielded pure nucleic acid. After drying, DNA was dissolved in TE-buffer.

Human DNA was prepared from peripheral blood mononuclear cells (PBMC). After separation from whole blood by Ficoll-Hypaque (Sigma) PBMC were incubated overnight with sarcosyl (Sigma) 0·03% and proteinase K (Boehringer Mannheim, Mannheim, Germany) 0·4 mg/ml at 55°C. DNA was purified by phenol–chloroform extraction and ethanol precipitation.

PCR amplification

For the amplification of the *M. leprae*-specific repetitive sequence, a set of primers was synthesised: ML-1 (GCACGTAAGCCTGTCGGTGG) and ML-2 (CGGCCGGATCCTCGATGCAC), according to Woods and Cole.[2] Oligonucleotides were synthesised in our laboratory by means of an automated DNA synthesiser model 381A (Applied BioSystems, ABI, Foster City, USA), with β-cyanoethyl phosphoramidite chemistry. Oligonucleotides were purified by use of TLC or on Oligonucleotide Purification Cartridges (OPC; ABI).

All PCR amplifications were performed in a Thermal Cycler model 480 (Perkin-Elmer-Cetus, Norwalk, CT, USA). A given quantity of template DNA (10 ng in most experiments) was mixed with 200 ng of each primer, 1 U of *Taq* polymerase (Ampli-Tac, Perkin-Elmer-Cetus), 200 μM of each deoxynucleotide triphosphate (stock solution 5 mM, pH 8·3), 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, gelatin 0·01% and water to a final volume of 50 μl. The mixture was overlaid with 50 μl of mineral oil (Perkin-Elmer-Cetus), and samples were held at 92°C for 3 min to denature chromosomal DNA, followed by 30 or 45 cycles of 2·5 min at 55°C, 2 min at 72°C and 1·5 min at 92°C, and a final extension cycle at 72°C for 7 min. Samples were analysed by gel eletrophoresis in an agarose 3%: Nusieve gel (1:2; purchased from Sigma and FMC Bioproducts, Oakland, USA, respectively). DNA in the gel was visualised by ethidium bromide staining.

DNA transfer and Southern hybridisation

An oligonucleotide ML-97 (TTTTAGTGTG CATGTCATGG), complementary to the amplified fragment of the repetitive sequence, or a 742-bp *Bss* HII fragment containing part of the repetitive sequence (obtained from a plasmid containing the gene coding for the 65-kDa antigen and part of its 3′ flanking sequence,[13] kindly provided by Dr B. Jacobs, Albert Einstein College of Medicine, NY, USA) was used for hybridisation. DNA was denatured by soaking the gel in 0·4 N NaOH for 10 min, and transferred to a nylon membrane (Zeta-Probe, BioRad, Richmond, USA) overnight by alkaline capillary blotting. Membranes were pre-hybridised for 3 h in 6 × SSPE (20 × SSPE is 3·6 M NaCl, 200 mM NaH_2PO_4 and 20 mM EDTA, pH 7·4), 5 × Denhardt's solution and SDS 0·5% at 45°C. When the 742-bp fragment was used as a probe, formamide 50% was added to the solution. Membranes were hybridised overnight at 42°C in the same solutions, adding 10^6 cpm/ml of either the oligonucleotide that was radiolabelled with ^{32}P-ATP (ICN Inc., Irvine, USA) to a specific activity of 2×10^7 cpm/μg,[14] or the 742-bp fragment that was ^{32}P-labelled by random priming, to a specific activity of 6×10^7 cpm/μg.[14] All membranes were washed with 2 × SSPE and SDS 0·1% at 42°C for 30 min; a second washing for the same period was carried out either in the same solution at 55°C (oligonucleotide), or in 0·5 × SSPE and SDS 0·1% at 68°C (742-bp fragment).

Collection and processing of clinical samples for PCR

Minute fractions of blood, lymph and biopsy material, initially from multibacillary, and later also from paucibacillary, untreated leprosy patients, were collected in the Souza Araujo ambulatory of the Oswaldo Cruz Foundation as part of the normal diagnostic routine.[15,16] All patients were clinically well defined and the bacillary index (BI) and morphological index (MI) in biopsy and lymph samples was determined. For PCR analysis, fractions of the original samples were further processed as follows: a small piece (about 1 mm^3) of biopsy material was incubated with 50 μl of 0·5 N NaOH at room temperature for

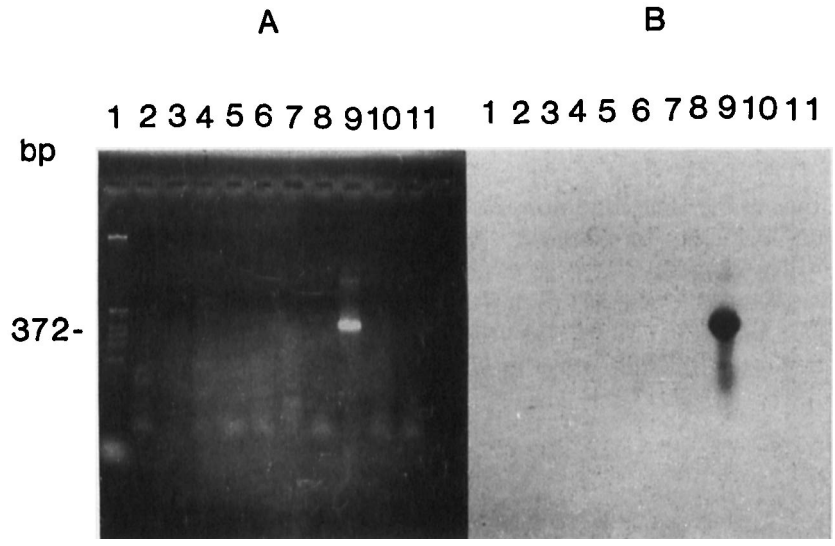

Fig. 1. *M. leprae*-specific amplification (30 cycles) as shown by visual (**A**) and hybridisation (**B**) analysis. Lane **1**, pBR322-*Hinf* I; **2**, 10 ng of purified DNA from *M. szulgai*; **3**, *M. bovis*-BCG; **4**, *M. kansasii*; **5**, *M. tuberculosis* $H_{37}Ra$; **6**, *M. tuberculosis* $H_{37}Rv$; **7**, *M. smegmatis*; **8**, *M. avium*; **9**, *M. leprae*; **10**, human DNA; **11**, a negative control without target DNA. **B**, Amplified material was hybridised with the 742-bp fragment containing the *M. leprae* specific repetitive sequence.

10 min with occasional mixing, neutralised with 1 M NaH_2PO_4, centrifuged, and the supernate was removed. Further treatment was done with two different protocols. In protocol I, modified from Hermans *et al.*,[6] the pellet was resuspended in 50 μl of a solution of 50 mM Tris-HCl and 5 mM EDTA (pH 8·0), incubated with lysozyme (Sigma) 1 mg/ml for 90 min at 37°C, and followed by incubation with proteinase K 1 mg/ml and SDS 1 % for 30 min at 60°C and stored at 4°C. In protocol II, adapted from Sritharan *et al.*,[17] the pellet obtained as described above was resuspended in 50 μl of TE-buffer containing Triton X100 1 % (TE-Triton), and stored at 4°C. After initial processing and before testing in PCR, both types of samples were submitted to a thermic shock procedure, consisting of three consecutive cycles of 10 min boiling and snap-freezing. Samples were then stored at −20°C.

Blood (1·5 ml) was mixed with the same volume of phosphate-buffered saline (PBS; pH 7·2), after which PBMC were isolated on a Ficoll-Hypaque density gradient (Sigma). Isolated PBMC were washed twice with PBS, pre-treated with 0·25 N NaOH, neutralised with 1 M NaH_2PO_4, centrifuged, resuspended in 40 μl of TE-Triton, and submitted to thermic shock as described above.

Lymph (1 or 2 μl), collected from the ear lobe, was mixed with 40 μl of TE-Triton and submitted to the thermic shock procedure as described above.

Results

Specificity of amplification of M. leprae DNA

With experimental conditions similar to those of Woods and Cole,[2] purified DNA from *M. leprae*, from seven other species of mycobacteria, and human DNA was submitted to PCR amplification. Only DNA from *M. leprae* gave the expected amplification product of 372 bp (fig. 1A). Amplified DNA was hybridised with the oligonucleotide probe ML-97, and, as shown in fig. 1B, hybridisation of the probe occurred only with amplified *M. leprae* DNA. In some experiments, a slight non-specific background amplification of human DNA was obtained; however, no hybridisation signals were found with this amplification product (data not shown).

Sensitivity of the PCR assay

Samples from a serial dilution of *M. leprae* DNA were added to the PCR reaction mixture and amplified during 45 cycles. Staining of amplified material with ethidium bromide revealed a detection limit of 100 ag (fig. 2). Given that the size of the *M. leprae* genome is $2{\cdot}2 \times 10^9$ Da,[18] 100 ag of DNA corresponds to *c.* one-tenth of the bacterial genome.

Amplification of M. leprae DNA in clinical samples from multibacillary leprosy patients

Four pieces of a skin biopsy from a patient with multibacillary leprosy and three pieces from a patient with a non-leprosy dermatosis (polymorphous light eruption) were processed by two different protocols as described in *Materials and methods*, and submitted to amplification by PCR involving 30 and 45 cycles. Fig. 3 clearly shows that the protocol with lysozyme–proteinase K-SSD, followed by thermic shock, was less efficient than submitting the biopsy material to thermic shock in the presence of Triton X100 1 %. Also, the non-specific amplification of human DNA was less after processing with the latter protocol. Hybridisation of the oligonucleotide probe or the 742-bp fragment occurred only with amplified DNA from infected patients (data not shown). As thermic shock in the presence of a small amount of Triton X100 gave

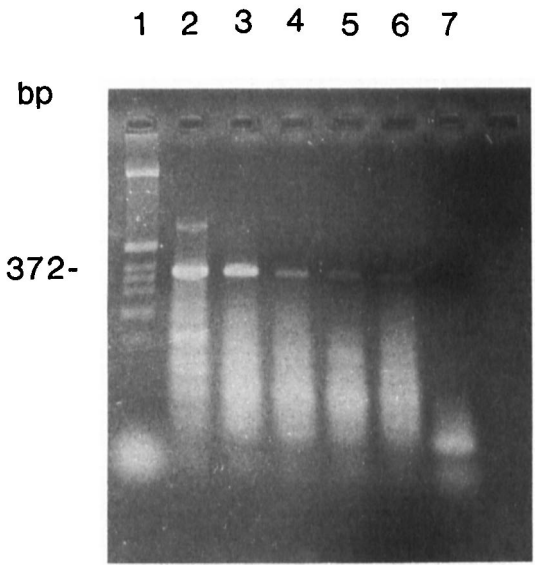

Fig. 2. PCR amplification (45 cycles) of a dilution of purified *M. leprae* DNA. Lane **1**, pBR322-*Hinf* I as a mol. wt marker; **2**, 10 ng of DNA; **3**, 100 pg; **4**, 100 fg; **5**, 1 fg; **6**, 100 ag; **7**, a negative control without target DNA.

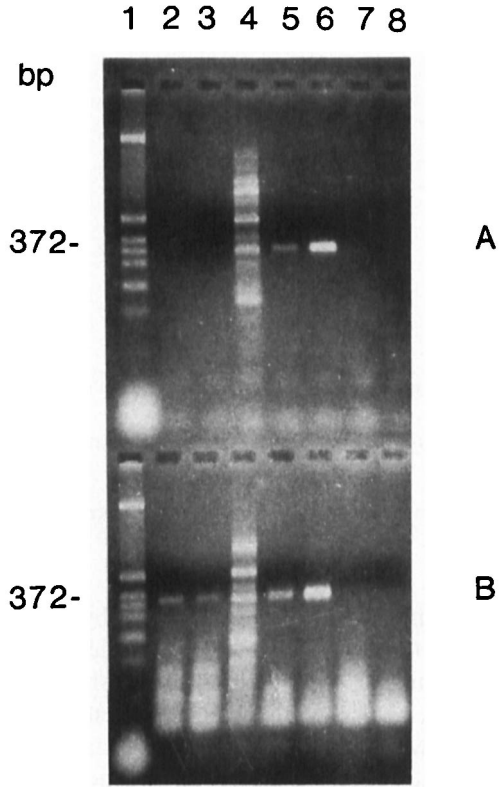

Fig. 3. Specific amplification of *M. leprae* DNA in a biopsy sample from a multibacillary leprosy patient (**2A-B, 3A-B, 5A-B** and **6A-B**) and a patient with polymorphous light eruption (**4A-B, 7A-B** and **8A-B**), after processing of the biopsies with protocol I (**2A-B, 3A-B** and **4A-B**) or protocol II (**5A-B, 6A-B, 7A-B** and **8A-B**). Lane **1**: pBR322-*Hinf* I mol. wt marker. Five μl of processed material was submitted to amplification for 30 (**A**) or 45 (**B**) cycles.

satisfactory results, and this treatment has been reported to function in other PCR systems,[16] it was applied to other types of clinical samples.

After thermic shock treatment of PBMC, isolated from 1·5 ml of blood from an LL patient and from one normal control, one-twentieth of the material was amplified through 45 cycles. Lymph, collected

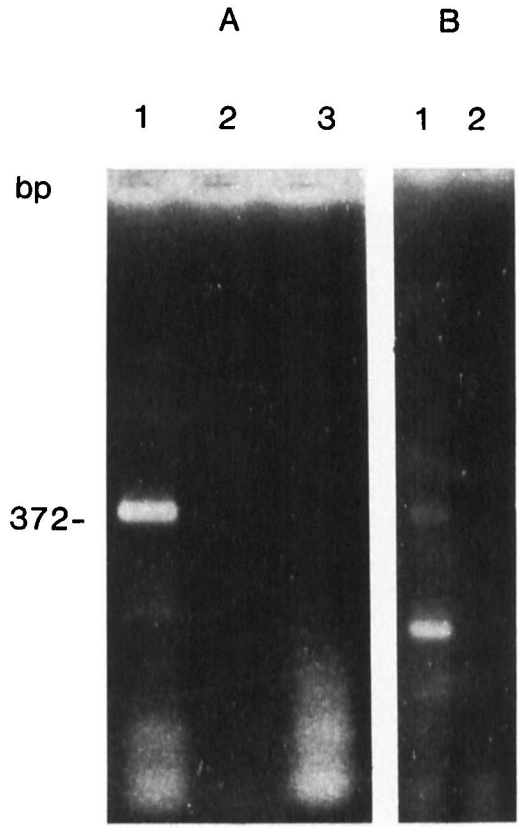

Fig. 4. Specific amplification of *M. leprae* DNA in lymph (**A**) and PBMC (**B**) samples from multibacillary leprosy patients (**A1** or **B1**) or normal donors (**A2–3** or **B2**); 2 μl of processed PBMC (**B1-2**) and 1 μl (**A1–3**) of lymph were submitted to 45 cycles of PCR.

from the ear lobe of a patient with multibacillary leprosy and from two normal controls, was submitted to thermic shock, and one-fiftieth and one-tenth of this material was also amplified in this way. Although a lower mol. wt band with stronger intensity was prominent in the amplification product from blood-derived material, amplification of the 372-bp fragment was clearly visible in the sample from the leprosy patient (fig. 4B). After blotting, the probes hybridised only with the 372-bp fragment of the amplified sample from leprosy patients. In contrast to the results obtained with blood samples, only a 372-bp product was detected after amplification of lymph material. As with PBMC, hybridisation experiments were positive only for amplified material from leprosy patients (data not shown). Surprisingly, no amplification product was seen with 5 μl of lymph solution. Reconstitution experiments with purified *M. leprae* DNA revealed that final Triton X100 concentrations of $> 0{\cdot}04\%$ strongly inhibited amplification. Unrelated to the action of Triton X100, thermic shock-treated PBMC also inhibited PCR amplification. However, a simple pre-treatment with NaOH, as described above, neutralised this inhibitory activity and allowed the use of 5 μl of processed PBMC in the PCR mixture.

Diagnosis of leprosy

The presence of *M. leprae* in biopsy material, blood

Table. Diagnosis of leprosy in patients with different clinical forms of the disease by PCR and subsequent hybridisation detection

Patient no.	Clinical information: Disease classification	Bacterial index: LE	LY	GBI	PCR results: Biopsy vis	Biopsy hyb	Lymph vis	Lymph hyb	Blood vis	Blood hyb
1	LL	4	3	4		ND		ND	–	–
2	LL	5	4	4		ND		ND	+	+
3	LL	4	5	5		ND		ND	+	+
4	LL	6	5	5		ND	–	?	–	+
5	LL	5	4	5	+	ND		ND	–	ND
6	LL	5	4	5	–	+	+	?	+	+
7	LL	4	5	4	+	ND	–	+	–	ND
8	LL	3	4	3		ND	–	?	+	–
9	BL	0	2	2		ND		ND	–	–
10	BL	4	4	4	+	+	–	+	ND	
11	BL	5	1	4	+	+		ND	–	–
12	BL	2	1	2	+	+		ND	ND	
13	BL	3	4	4	–	+	–	+	ND	
14	BL	4	2	3	–	+†	+	+	ND	
15	BL	4	0	3	+	+	+	?	+	–
16	BL	4	3	4	+	+		ND	ND	
17	BB	2	3	2		ND	–	+	+	–
18	BB	4	3	3	–	+†	–	+	+	+
19	BB	3	0	3		ND	–	?	–	+
20	BT	0	0	0		ND	+	?	+	ND
21	BT	0	0	0	+	+	–	?	–	–
22	BT	0	0	0	+	+	–	+	+	–
23	BT	0	0	0	+	+	–	+	ND	
24	BT	0	0	0	+	+	–	+	ND	
25	BT	0	0	0		ND	–	+	ND	
26	TT	0	0	0	+	+	–	+	ND	
27	I	0	0	0	+	+	–	+	ND	
	Healthy controls									
	1	0	0	0	–	–				
	2						–	–		
	1								–	–

LL, lepromatous leprosy; BL, borderline lepromatous leprosy; BB, borderline leprosy; BT, borderline tuberculoid leprosy; TT, tuberculoid leprosy; I, indeterminate leprosy.
The bacterial index was determined in the lesion biopsied (LE), in the lymph (LY) and as the mean value of six sites, the general bacterial index (GBI).
ND, not determined.
+, positive; –, negative; ?, doubtful; vis, by direct visualisation of a product by ethidium bromide staining; hyb, by Southern hybridisation.
† These results were initially negative but were subsequently found to be positive after an additional DNA extraction.

or lymph samples from a total of 27 untreated leprosy patients with various forms of the disease was analysed. Eight lepromatous leprosy (LL) patients, eight borderline lepromatous (BL) patients, three borderline leprosy (BB) patients, six borderline tuberculoid (BT) patients, one tuberculoid (T) and one indeterminate (I) patient, classified according to the Ridley and Jopling scale,[19] were included in the study. Samples were processed and submitted to PCR, followed by both visual and hybridisation analysis of the amplified material (table). PCR of samples from normal donors or patients with non-leprosy skin diseases (polymorphous light eruption or discoid lupus erithematosus) gave negative results in all experiments. Amplified material was hybridised with the internal oligonucleotide M1-97 or with the 742-bp fragment, or both, and results were considered to be positive when at least one gave a hybridisation signal.

All but two patients from whom biopsy material was amplified were found to give positive results after PCR amplification—13 (76%) of 17 by visual detection; 13 (87%) of 15 by hybridisation. The two negative samples were subsequently shown to be positive after PCR of the DNA extracted from the biopsy material with a very efficient but somewhat laborious extraction protocol for mycobacterial DNA, described earlier.[12] Several lymph samples, although negative upon visual evaluation (positivity 4 of 19, 21%), were positive after hybridisation (13 of 19, 68%). In some of these experiments (6 of 19), a faint hybridisation signal was observed with the mol. wt marker when the 742-bp fragment was used as a probe. When this occurred, hybridising samples were not considered to be positive. Results obtained with blood samples were sometimes difficult to evaluate. Overall positivity—9 (53%) of 17 by visual detection; 6 (43%) of 14 by hybridisation—in blood samples was lower than that found in other samples; also, some samples which seemed positive by visual evaluation, did not show any hybridisation signal.

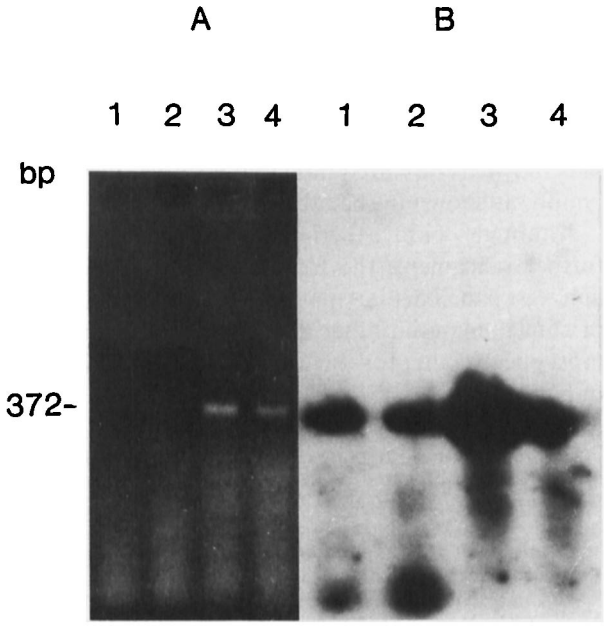

Fig. 5. Amplification of *M. leprae* DNA in processed biopsies from a BB leprosy patient (lanes **1** and **2**) and from a BL leprosy patient (**3** and **4**). Either 5 μl (**1** and **3**) or 1 μl (**2** and **4**) were submitted to 45 cycles of PCR and analysed visually (**A**) or after hybridisation with the 742-bp fragment (**B**).

Discussion

One of the first reports on the use of PCR as a potential tool for diagnosis of leprosy described an *M. leprae*-specific repetitive sequence as a target for amplification.[2] Because of the high number of copies of this repetitive sequence present in the genome, and because of the specificity of this PCR system, the amplification conditions were optimised and the applicability of this system as a diagnostic tool for leprosy, with different types of clinical samples was investigated. To eliminate false positive results due to non-specific amplification, amplified material was hybridised with an internal probe or with a fragment of the cloned repetitive sequence. In this way, the specificity of the amplification of *M. leprae* DNA was confirmed. Forty-five cycles of PCR amplification of purified *M. leprae* DNA revealed as little as 100 ag of target DNA, an amount equal to *c.* one-tenth of the bacterial genome.[18] The sensitivity obtained here was at least 10 times higher than described previously for *M. leprae*[3,4] and approaches the theoretical detection limit of this method.

So far, few studies have shown experimental data on PCR detection of *M. leprae* in clinical samples, amplifying only mycobacterial DNA in frozen and fixed sections of biopsy material from leprosy patients.[7,10] As patients do not always present with lesions and detection of the leprosy bacillus in clinical material obtained by less invasive procedures is desired, we tried to extend the diagnosis of leprosy by the investigation of different types of clinical samples. Standardisation of sample processing was initiated with fragments from skin biopsies. Woods and Cole reported a simple freeze-boiling method for *M. leprae* DNA extraction and subsequent PCR,[2] and recently, the addition of Triton X100 during this process was shown to induce lysis of 95% of *M. tuberculosis* bacilli.[17] Treatment of biopsy fragments from LL patients with this modified protocol resulted in detection of *M. leprae* DNA after only 30 PCR cycles. Furthermore, this method was found to be more efficient than treatment of biopsy samples with lytic enzymes, a process that seems to function well for the detection of *M. tuberculosis* in sputum.[6] Because of its simplicity and efficiency, the former method for processing different clinical samples was adopted, although the possibility that more efficient protocols for the detection of *M. leprae* may be found cannot be excluded. During the preparation of this manuscript, sonication was reported to be more efficient for extracting DNA from clinical samples containing *M. tuberculosis* then freeze-boiling processes;[20] however, inhibition of PCR by some components from the resulting crude lysate could be the main drawback of this procedure. Inhibition of PCR by components of various clinical samples and especially of blood has been reported,[21] and has also been described for detection of *M. leprae*.[7] We observed inhibition of amplification in experiments with PBMC but inhibitory activity could be neutralised by a simple treatment of the PBMC samples with 0·5 N NaOH. This procedure was used routinely and allowed the application of more sample material to the PCR reaction.

Although we do not consider our sample processing conditions to be fully optimised (experiments with sonication as a treatment are being examined), the value of the PCR conditions was assessed on samples from patients. Patients from the opposite poles of the leprosy spectrum were screened for the presence of *M. leprae* in at least one type of clinical sample. The number of positive results found in biopsy and lymph samples from paucibacillary patients was encouraging, and clinical material from non-leprosy donors did not yield any false positive results. Several patients in whom microscopic examination did not reveal any bacteria gave positive results with PCR. This is in agreement with results obtained by De Wit *et al.*,[7] who amplified bacterial DNA in sections of biopsy samples taken from paucibacillary and from treated leprosy patients; however, they reported the appearance of false positive results with their methodology. In the two cases in which bacteria were not detected in biopsy material, an additional extraction of the DNA from the processed biopsy sample and subsequent PCR showed the presence of *M. leprae* DNA. Also, patients with a positive bacterial index (BI) but with a morphological index (MI) of zero were found to be PCR-positive in our study. It is well established that the determination of BI and MI is variable, and that both are relatively insensitive techniques.[22] Also, the correlation between morphology and viability of *M. leprae* is not universally accepted,[23] and the question has arisen as to whether PCR can be positive

after killing of bacteria and disposal of DNA from the host tissue.[24] Several of the untreated LL patients who gave positive results in the PCR had an MI equal to zero; however, it is highly unlikely that these patients did not harbour any viable bacteria. Whatever the relationship between positivity of PCR and viability of *M. leprae*, PCR is much more sensitive than microscopic examination for direct detection of bacilli.

Although we consider these results to be very encouraging, some experimental drawbacks still have to be resolved. Amplification of *M. leprae* in blood samples gave inferior results in comparison to those with other types of clinical material. Although the presence of bacteria in blood samples, contrary to the case with lymph and biopsy samples, was not shown by microscopical examination, it is likely that sufficient amounts of bacteria are present in blood of at least LL patients to allow PCR detection.[25] The analysis of blood samples yielded some non-specific amplification products, so additional steps in the blood processing protocol will have to be developed. The cloned 742-bp fragment needs improvement as a probe. In some cases, the pBR322 *Hinf* I mol. wt marker gave a slightly positive signal after hybridisation with the 742-bp restriction fragment, which is probably due to some contamination of the probe preparation with plasmid. This obscured potential positive signals with lymph, as shown in the table.

Although our experimental protocol requires further refinement, the frequency of PCR positivity amongst paucibacillary patients and the successful use of clinical material other than skin biopsy samples is most encouraging for the use of PCR in the diagnosis of leprosy.

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