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Application of a Polymerase Chain Reaction for the Detection of *Mycobacterium leprae* in Skin Tissues

MADELEINE Y. L. DE WIT,[1] WILLIAM R. FABER,[2] SUZE R. KRIEG,[3] JAMES T. DOUGLAS,[4] SEBASTIAN B. LUCAS,[5] NIWAT MONTREEWASUWAT,[6] STEFAAN R. PATTYN,[7] RABIA HUSSAIN,[8] JORG M. PONNIGHAUS,[9] RUDY A. HARTSKEERL,[1] AND PAUL R. KLATSER[1]*

N. H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Meibergdreef 39, 1105 AZ Amsterdam Z.O.,[1] and Departments of Dermatology[2] and Pathology,[3] Academic Medical Centre, University of Amsterdam, 1012 WX Amsterdam, The Netherlands; Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822[4]; Department of Histopathology, University College, Middlesex Hospital, London WC1 6BT, United Kingdom[5]; Leprosy Division, Raj-Pracha-Samasai Institute, 10130 Samutprakarn, Thailand[6]; Department of Microbiology, Institute for Tropical Hygiene, 2000 Antwerp, Belgium[7]; Department of Microbiology, Aga Khan Medical College, Karachi 74800, Pakistan[8]; and LEPRA Evaluation Project, Chilumba, Karonga District, Malawi[9]

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The polymerase chain reaction (PCR) based on the selective amplification of a 530-bp fragment of the gene encoding the proline-rich antigen of *Mycobacterium leprae* was applied on sections of fixed or frozen biopsy samples from leprosy patients. A simple procedure for the extraction of DNA from *M. leprae* in clinical specimens that provided suitable template DNA for amplification was developed. When PCR was applied on frozen sections, positive amplification in samples from all untreated acid-fast bacillus (AFB)-positive patients and in samples from 56% of the untreated AFB-negative patients could be detected, while biopsy samples from patients with skin diseases other than leprosy were all PCR negative. With neutral Formalin-fixed biopsy samples, positive amplification in 92% of the samples from untreated AFB-positive patients and in 61% of the samples from untreated AFB-negative patients could be detected by PCR. Biopsy samples exposed to mercuric chloride or nonbuffered formaldehyde containing fixatives were not suitable for application of PCR. This PCR holds promise as a tool for studies on *M. leprae* infection.

Methods to detect and quantify *Mycobacterium leprae* are greatly needed for studies involving the epidemiology, pathogenesis, and chemotherapy of leprosy. Serological assays (2) and skin tests (12) lack the required sensitivity and specificity to serve as diagnostic tools for *M. leprae* infection. Even when satisfactory immunodetection is achieved, it may just be a reflection of a past infection, giving no information on the current bacteriological status.

Previously, we described a polymerase chain reaction (PCR) using heat-stable *Taq* polymerase for the specific detection of *M. leprae* (3). A set of primers was selected on the basis of the nucleotide sequence of a gene encoding the proline-rich antigen of *M. leprae* (3, 9). With this set of primers in the PCR, *M. leprae* could be detected specifically with a detection limit approximating one bacterium when using suspensions of bacilli. On the basis of these initial experiments using purified bacilli, this PCR appeared to meet the criteria of specificity and sensitivity required for a useful tool for the detection of *M. leprae*. Such a method of detection would alleviate the inability to cultivate *M. leprae*, which has hampered the investigation of the distribution and spread of the bacillus in a given population.

The usefulness of this PCR for the detection of infection requires further evaluation in well-defined populations. Considering the lack of an independent "gold standard" for the diagnosis of leprosy, clinical findings, accompanied by the demonstration of leprosy bacilli in skin through acid-fast staining in conjunction with appropriate patterns of inflammation in that tissue, remain the only available standard for the diagnosis of leprosy. In this study, we applied the PCR on both fresh frozen and fixed paraffin-embedded skin biopsy samples from leprosy patients diagnosed according to the above standard, allowing evaluation of the PCR in relation to the available routine of diagnostic methods.

* Corresponding author.

MATERIALS AND METHODS

Skin biopsies. The study patients resided in Malawi, Pakistan, Thailand, Belgium, and the Netherlands. Leprosy patients were classified clinically and histopathologically according to the Ridley-Jopling scale (8) as lepromatous, borderline lepromatous, borderline, borderline tuberculoid, tuberculoid, or indeterminate. Punch skin biopsy samples were taken from skin lesions of leprosy patients and patients with skin diseases other than leprosy, according to standard methods. Biopsy samples were either quick-frozen in liquid nitrogen or fixed. Three fixatives were used: Formalin, which consisted of 4% (vol/vol) formaldehyde in 0.9% (wt/vol) NaCl; neutral Formalin, which consisted of 4% (vol/vol) formaldehyde in 0.35% (wt/vol) $NaH_2PO_4 \cdot 2H_2O$ and 0.65% (wt/vol) $Na_2HPO_4 \cdot 12H_2O$; and FMA (formaldehyde-mercuric chloride-acetic acid), which consisted of 4% (vol/vol) formaldehyde, 2% (wt/vol) $HgCl_2$, and 3% (vol/vol) acetic acid. Biopsy samples were incubated for 2 h and then transferred to 70% (vol/vol) ethanol. The biopsy samples originated from the patients residing in the Netherlands (frozen biopsy samples), in Belgium (Formalin-fixed biopsy samples), in Thailand (neutral Formalin-fixed biopsy samples), or in Malawi or Pakistan (FMA-fixed biopsy samples).

Enumeration of acid-fast bacilli was assessed by means of the bacterial index (BI) of the granuloma on sections stained by the modified Fite method (7).

From either type of biopsy sample 5-μm sections were

used for PCR. All samples were coded, and PCR was performed without knowledge of the classification of the sample.

Liver tissue samples were collected from experimentally *M. leprae*-infected and noninfected armadillos, and the samples were quick-frozen and handled in the same way as human skin biopsy samples.

Preparation of chromosomal DNA. *M. leprae* DNA was purified from armadillo-derived bacilli as described previously (3) and was used as a positive control in all PCR experiments.

Frozen sections (5-μm thickness) were incubated with 50 μl of 100 mM Tris-HCl, pH 8.5, containing 0.05% Tween 20 and 60 μg of proteinase K per ml for 18 h at 60°C. Paraffin oil (40 μl) was layered on top to prevent evaporation. Thereafter, the samples were incubated at 97°C for 15 min. Unless otherwise indicated, 25 μl of the sample was then tested in PCR. PCR-negative specimens were retested by using 10, 5, or 2 μl of the sample in the amplification mixtures.

Fixed paraffin-embedded tissue sections (5-μm thickness) were deparaffinized with 400 μl of xylol and washed twice with 800 μl of 96% (vol/vol) ethanol and once with 800 μl of acetone, after which the tissue pellet was allowed to dry. This pellet was then treated identically as the frozen sections.

PCR. Amplification was performed with the thermostable *Taq* DNA polymerase (purchased from Perkin-Elmer Cetus) as described previously (3), with some modifications. Briefly, samples to be amplified were incubated in a 50-μl reaction volume containing 10 mM Tris-HCl (pH 9.6), 50 mM NaCl, 0.01% gelatin, 12.5 mM $MgCl_2$, 200 ng each of oligonucleotide primers S13 and S62 (3), 1 mM (each) dATP, dCTP, dGTP and dTTP, and 2.5 U of *Taq* DNA polymerase. The reaction mixtures were covered with 40 μl of paraffin oil. Rounds of amplification consisted of a 2-min denaturation step at 94°C, a 2-min annealing step at 55°C, and a 3-min elongation step at 72°C. After the 32nd cycle, the samples (20 μl) were analyzed by electrophoresis on 2% (wt/vol) agarose gels for the presence of bands of the appropriate size (530 bp) and amplification of the target DNA was confirmed by subsequent Southern blotting with a 1.0-kb *Eco*RI fragment comprising the gene encoding the proline-rich antigen of *M. leprae* as a DNA probe, as described before (3). Positive controls of 625 pg, 62.5 pg, 6.25 pg, 625 fg, and 62.5 fg of purified *M. leprae* DNA and five negative controls with no target DNA (e.g., proteinase K-Tween 20 buffer) were run in parallel with the clinical samples. A tissue specimen was considered positive when, at any dilution of the sample (see above), analysis by both agarose gel electrophoresis and subsequent Southern blotting revealed a visible 530-bp fragment in the amplified material.

RESULTS

A simple method for the extraction of DNA from clinical samples was developed. This method involved proteinase K-Tween 20 treatment of the sample without any further DNA purification. Previously, we described a DNA extraction method which included DNA purification in addition to an elaborate enzyme treatment of the sample (3). As shown in Fig. 1, no difference in amplification efficacy between the two methods was found when PCR was applied on frozen liver tissue sections from infected armadillos.

By using this simple DNA extraction method, PCR was applied on frozen and fixed biopsy sections from leprosy patients and controls. As shown in Fig. 2, frozen sections which were initially negative by PCR could test positive when the amount of sample was reduced in the amplification mix (Fig. 2, lane 2). Similar results were found with fixed biopsy sections (result not shown). Therefore, we retested all negative samples by decreasing the sample volume from 25 to 10, 5, or 2 μl.

Table 1 shows the result of PCR applied on FMA-fixed biopsy sections from 31 untreated leprosy patients and 4 nonleprosy controls. Only 67% of the biopsy sections, which

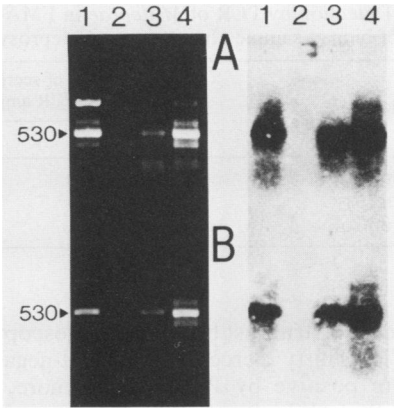

FIG. 1. Agarose gel (left) and Southern blot (right) analyses of PCR-amplified products of samples which contained DNA extracted from purified *M. leprae* (lane 1) and from frozen liver tissue sections from uninfected armadillos (lane 2) and from experimentally infected armadillos which contained 10^7 (lane 3) and 2×10^{10} (lane 4) *M. leprae* (g of liver tissue^{-1}). Two methods of target DNA extraction were applied: lytic enzyme treatment (3) (A) and proteinase K treatment (see Materials and Methods) (B). Numbers at left indicate sizes (in base pairs).

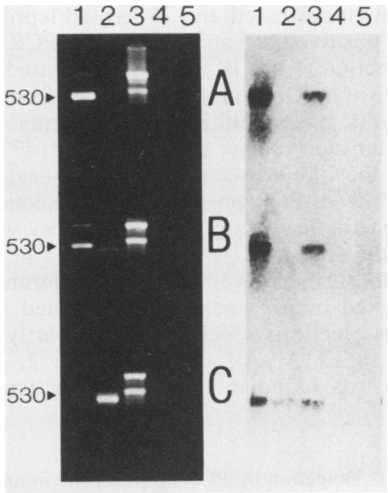

FIG. 2. Agarose gel (left) and Southern blot (right) analyses of PCR-amplified products of samples which contained DNA extracted from sections of FMA-fixed and frozen biopsy samples from the same patients. Frozen (lane 2) and FMA-fixed (lane 4) sections of a biopsy sample from a patient with multibacillary leprosy. Frozen (lane 3) and FMA-fixed (lane 5) sections of a biopsy sample from another patient with multibacillary leprosy. Lane 1 contains PCR-amplified products from a sample which contained 62.5 pg of target DNA purified from *M. leprae*. Amplification mixtures contained 10 μl (A), 5 μl (B), or 2 μl (C) of the samples in the amplification mix.

TABLE 1. Detection by PCR of *M. leprae* in FMA-fixed tissue sections of biopsy samples from untreated leprosy patients

Patient classification	No. of sections showing PCR amplification	
	+	−
BI-positive leprosy	14	7
BI-negative leprosy	3	7
Nonleprosy control	1	3

showed staining of acid-fast bacilli by microscopy, showed a positive PCR. Thirty percent of the BI-negative biopsy sections were positive by PCR. Furthermore, one of the nonleprosy control biopsy sections (a case of syphilis) showed positive amplification.

Table 2 summarizes the results obtained with unbuffered Formalin-fixed biopsy sections from 39 untreated leprosy patients and 5 nonleprosy patients. Of the BI-positive sections, 50% showed positive amplification after PCR, and of the BI-negative sections, 77% showed positive amplification. Of the five nonleprosy patients, three showed positive amplification after PCR.

Table 3 shows the results of PCR applied on neutral Formalin-fixed biopsy sections from both untreated and treated leprosy patients (n = 48). Ninety-two percent of the biopsy sections from BI-positive untreated patients were PCR positive. The one patient whose biopsy section was PCR negative was diagnosed with indeterminate leprosy and had a BI of 1. Of the biopsy sections from untreated patients in which no acid-fast bacilli were detected by microscopy, 61% were PCR positive. Of the biopsy sections from patients undergoing therapy for different periods, 83% of those from BI-positive cases and 83% of those from BI-negative cases were PCR positive. One control biopsy section from a patient with seborrheic keratosis was PCR negative.

Table 4 shows the results of PCR applied on frozen biopsy sections from 20 untreated and 23 treated leprosy patients. All of the BI-positive untreated cases were PCR positive. Of the biopsy sections from BI-negative untreated cases, 56% were PCR positive. Of the biopsy sections from BI-positive treated patients (taken either while patients were under therapy or after their release from treatment) 57% were PCR positive. Of the biopsy sections from BI-negative treated patients, 38% were PCR positive. Biopsy sections from 11 patients with skin diseases other than leprosy were all PCR negative (Table 4).

In order to further investigate the difference between frozen and fixed biopsy sections, we applied PCR on BI-positive biopsy sections which were split, partly frozen, and partly fixed in FMA. As shown in Fig. 2, no positive amplification was found in two biopsy sections from two different patients, which were FMA-fixed (Fig. 2, lanes 4 and 5), although the corresponding frozen part of the same biopsy section did show amplification of the 530-bp fragment (Fig. 2, lanes 2 and 3).

TABLE 2. Detection by PCR of *M. leprae* in unbuffered Formalin-fixed tissue sections of biopsy samples from untreated leprosy patients

Patient classification	No. of sections showing PCR amplification	
	+	−
BI-positive leprosy	8	8
BI-negative leprosy	10	13
Nonleprosy control	3	2

TABLE 3. Detection by PCR of *M. leprae* in neutral Formalin-fixed tissue sections of biopsy samples from untreated and treated[a] leprosy patients

Treatment and BI	Patient classification[b]	No. of biopsy sections showing PCR amplification	
		+	−
Untreated			
Positive	I	0	1
	BL	5	0
	LL	6	0
Negative	I	6	3
	TT	2	2
	BT	3	2
Treated			
Positive	BT	0	1
	BL	3	0
	LL	2	0
Negative	TT	4	0
	BT	3	1
	BL	1	1
	LL	2	0

[a] Patients under therapy, receiving either multiple drug therapy according the World Health Organization regimen (11) or dapsone monotherapy.
[b] Patients were classified according to the Ridley-Jopling scale (8) as having indeterminate (I), tuberculoid (TT), borderline tuberculoid (BT), borderline lepromatous (BL), or lepromatous (LL) leprosy. A biopsy section of a patient with seborrheic keratosis was PCR negative.

DISCUSSION

Because *M. leprae* has not yielded to cultivation, the application of the PCR to the selective amplification of *M. leprae* DNA may be a breakthrough in leprosy research.

TABLE 4. Detection by PCR of *M. leprae* in frozen tissue sections of biposy sections from untreated and treated[a] leprosy patients

Treatment and BI	Patient classification[b]	No. of biopsy sections showing PCR amplification	
		+	−
Untreated			
Positive	BT	1	0
	BL	5	0
	LL	5	0
Negative	TT	1	1
	BT	4	3
Treated			
Positive	BL	1	2
	LL	3	1
Negative	BT	2	3
	BL	2	1
	LL	2	6
Nonleprosy controls		0	11

[a] Patients under therapy, receiving multiple drug therapy according the World Health Organization regimen (11), and patients after release from treatment.
[b] Patients were classified according to the Ridley-Jopling scale (8) as having indeterminate (I), tuberculoid (TT), borderline tuberculoid (BT), borderline lepromatous (BL), or lepromatous (LL) leprosy.

Previously, we described a PCR based on the selective amplification of a 530-bp fragment of the gene encoding the proline-rich antigen of *M. leprae* (3). Here, we report the validation of this assay with clinical samples.

We have established an alternative protocol for DNA extraction from *M. leprae* which is more simple than the extraction and purification method we described before (3), thus limiting the risk for contamination. This method gave essentially identical results when applied on frozen liver tissue sections from experimentally infected armadillos (Fig. 1). Such simple DNA extraction methods prior to amplification of *M. leprae* DNA, involving a single boiling step or repeated heat-cold shocks, have been described before (11).

When we applied this method of extraction on fixed or frozen tissue sections, we found that initially PCR-negative samples were rendered PCR positive by using reduced amounts of the sample in the amplification mix (Fig. 2). Since the DNA extraction method used does not involve any DNA purification steps, a possible reason could be that impurities in the extract inhibit the polymerase reaction. Sufficient dilution of the impurities would allow the amplification of the remaining DNA. This implicates a minor reduction in sensitivity compared with the previously reported detection limit approaching a single bacillus (3). Since a maximum dilution factor of 25 is applied, sensitivity should still be satisfactory for most samples and in any case much greater than that of the conventional method of microscopy (6).

When we applied PCR on tissue sections exposed to mercuric chloride or nonbuffered formaldehyde containing fixatives, both sensitivity and specificity were unsatisfactory (Tables 1 and 2). DNA recovered from samples exposed to mercuric chloride has been shown before not to be intact (1). In addition, formylation of nucleic acids produces Schiff bases on free amino groups of nucleotides (4), and cross-links between proteins and DNA may be formed (5). Although these processes are in principle reversible, increasing lengths of time of fixation in Formalin has been shown to decrease the amount of DNA suitable for hybridization (1). The DNA in these samples may thus have been unsuitable for amplification under the conditions used. This was substantiated by the results from one experiment in which we applied PCR on biopsy samples from two multibacillary patients, where part of each sample was frozen and the other part was FMA-fixed (Fig. 2). No positive amplification in the FMA-fixed sections could be detected, while in the corresponding frozen sections, positive amplification could be clearly detected.

False-positive amplification may have resulted from the many steps involved in processing, embedding, and sectioning. Usually, the same tissue-processing chambers are used for many biopsy samples, thus enhancing the risk for contamination. However, the section-cutting blades were cleaned between each sample, and the BI-negative samples were sectioned before the BI-positive blocks. Since the processing and sectioning techniques were similar for all three fixatives, one of which was not associated with false PCR amplification (although based on the one nonleprosy case available), the origin of the contaminant or false amplification remains to be assessed with certainty.

When we applied PCR on neutral Formalin-fixed or frozen skin biopsy sections, results were far better (Tables 3 and 4). Apart from the sample from one untreated indeterminate leprosy patient with a BI of 1, in all BI-positive biopsy sections, *M. leprae* DNA could be amplified. On reexamination of the histopathological slides of the neutral Formalin-fixed sections, we found in samples from patients with tuberculoid, borderline tuberculoid, or indeterminante leprosy an inverse correlation between the density of the cellular infiltrate, particularly numbers of lymphocytes in the section, and the degree of positivity by PCR (results not shown). The denser the infiltration by epithelioid cell granulomas or lymphohistiocytic aggregates in the sections, the less amplification found. This could be an indication that PCR positivity reflects the presence of viable bacilli at the time of biopsy. A strong host immune response could result in killing of *M. leprae* and breakdown or clearance of its DNA. A PCR-negative result in a biopsy section with a BI of 1, without knowledge on the viability of the bacilli, is thus well understandable. Woods and Cole (11) have also suggested that positive amplification reflects the presence of potentially viable *M. leprae*. On the other hand, cellular infiltrates may secrete some mediators which inhibit the PCR. Williams et al. (10), who applied a PCR on *M. leprae*-seeded normal skin biopsy material, have also suggested the presence of inhibitors in human skin.

Over half of the untreated BI-negative cases were PCR positive (Tables 3 and 4). It has been shown before that low densities of acid-fast bacilli in skin are underestimated or missed entirely by conventional microscopy, so that cases are missed as well as misclassified (6). In this respect, apparent identification of *M. leprae* DNA in tissue by PCR holds promise as an additional tool for the diagnosis of suspected cases of early leprosy.

When biopsy specimens from treated patients were examined, a larger percentage of PCR positives was found, both in BI-positive and BI-negative cases, in neutral Formalin-fixed sections than in frozen sections. This may be due to the fact that the frozen biopsy sections were also derived from patients after release for treatment (nine samples, of which only one was PCR positive), in contrast to the fixed biopsy samples, which were derived from patients during treatment only, including patients on dapsone monotherapy.

Several explanations can be put forward to explain PCR negativity. There may be no bacilli present at all, which is likely to be the case in some BI-negative cases, or bacilli may not be viable anymore, because of either a strong host immune response or killing by treatment. On the other hand, it cannot be ruled out that other factors, such as the age of the specimens, may also have an effect on the outcome of PCR. Further study is needed to evaluate the significance of the detection of *M. leprae* DNA in tissues of patients, especially during and after treatment.

Retrospective studies of a large number of patients could be envisaged if the vast amounts of material available in pathology laboratories throughout the world were suitable as DNA sources for PCR. However, as shown in this report, depending on the fixation procedure used, not all biopsy specimens are suitable for detection of *M. leprae* DNA by PCR, at least by our system of amplification. Nevertheless, PCR has been shown here to be a useful tool for the detection of *M. leprae* in neutral Formalin-fixed biopsy samples and especially in frozen biopsy samples. New insights into the dynamics of *M. leprae* infection and the pathogenesis of leprosy are likely products of this methodology.

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