Comparison of PCR mediated amplification of DNA and the classical methods for detection of *Mycobacterium leprae* in different types of clinical samples in leprosy patients and contacts


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Accepted for publication 4 November 2002

Summary  Traditional staining and microscopic examination techniques for the detection of *Mycobacterium leprae*, DNA amplification by polymerase chain reaction (PCR) of a 531-bp fragment of the *M. leprae* specific gene encoding the 36-kDa antigen, and serodiagnosis with *M. leprae* specific antigens (PGL-1 and D-BSA) were compared on different clinical specimens (serum samples, slit-skin smears, biopsies and swabs) from 60 leprosy patients attending the Sanatorium of Fontilles. Patients were divided into groups; (i) 20 multibacillary patients (MB) with positive bacteriological index (BI) by conventional methods and on WHO multidrug therapy (MDT); (ii) 30 MB patients with negative BI and completed minimum 2 years treatment MDT; (iii) 10 paucibacillary (PB) patients who had completed 6 months MDT at least 8 years ago. Control groups included four non-leprosy patients for PCR methods and 40 health control patients and 10 tuberculosis patients for serological methods. In the multibacillary BI positive group, there was a good correlation between all methods. All tests were negative in the paucibacillary group, although only a few patients were tested and all had been treated many years ago. One must be cautious concerning the diagnostic potential of these techniques in this type of leprosy. We also studied different combinations of leprosy diagnosis methods to determine the potential risk in a leprosy contact individuals group. The prevalence of antibodies to *M. leprae* antigens in serum was measured, together with the presence of *M. leprae* DNA in the nose and lepromin status in a group of 43 contacts of leprosy patients (12 household and 31 occupational) to evaluate the maintenance of infection reservoirs and transmission of the disease. Only two individuals were found to form a potential high risk group.

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Introduction

Leprosy is a chronic mycobacterial disease and its aetiological agent, *Mycobacterium leprae*, cannot be grown in vitro. The diagnosis and differentiation of leprosy from other cutaneous granulomatous diseases is routinely based on the presence of acid-fast bacilli (AFB) from a tissue smear previous staining with Ziehl–Neelsen or Fite–Faraco, techniques that are not *M. leprae* specific, since any AFB bacilli present in the sample will be stained, in combination with a clinical and histopathological evaluation. Because AFB staining techniques requires at least $10^4$ organisms per gram of tissue for reliable detection,¹ sensitivity is low, particularly for patients at the tuberculoid end of the leprosy spectrum where AFB are rare or absent and routine diagnosis is based on clinical symptoms and neural involvement and for patients being monitored for possible relapses after completing multidrug therapy (MDT).

The use of serological assays based on the employment of *M. leprae* specific antigens, principally phenolic glycolipid I (PGL-1) and its synthetic disaccharide (D-BSA), has opened new methods of monitoring *M. leprae* infections, since antibody levels are associated with intensity of exposure and systemic involvement to this bacteria.² However, serological assays do not reflect all clinical infections, since the majority of paucibacillary patients have not developed significant levels of antibody responses.³ Long-term follow-up studies⁴⁵ have detected new leprosy cases in seronegative groups, indicating that not all clinical and subclinical infections can be detected by a serological response.

New molecular biology methods have been developed as reliable and sensitive diagnostic tools for the identification of pathogens in many infectious diseases, with the specific polymerase chain reaction (PCR) amplification of *M. leprae* DNA the most used for detection and diagnosis of leprosy due to its sensitive, specific and rapid detection of microorganisms in clinical specimens. Many reports exist in the literature regarding the utility of different target sequences for PCR and DNA probes specific for *M. leprae*, such as genes encoding the 36-kDa antigen,⁶ ⁷ the 18-kDa antigen⁸ or the 65-kDa antigen⁹ and repetitive sequences of *M. leprae*.¹⁰¹¹

This technique can also be applied to the study of the transmission of leprosy and maintenance of infection reservoirs by detection of *M. leprae* DNA in the nasal cavity, since this route is involved in the carriage and shedding of *M. leprae¹²¹³ and the nose is also considered the port of entry of the bacilli.¹⁴

The aim of this study was to evaluate the usefulness of the PCR method on several clinical specimens, compared with more conventional methods (slit-skin smears and histopathological sections) and including the humoral response to *M. leprae* specific antigens PGL-1 and D-BSA, on a group of leprosy patients divided into (i) multibacillary patients (MB) with positive bacterial indices (BI) and still on WHO recommended multidrug therapy (MDT); (ii) MB patients with negative BI and who completed MDT; (iii) paucibacillary patients (PB) who had completed 6 months WHO MDT.

The second purpose of the study was to evaluate nasal carriage of *M. leprae* DNA by PCR on nasal swabs, together with prevalence of humoral responses to *M. leprae* specific antigens (PGL-1 and D-BSA) and lepromin status, on a group of contacts of leprosy patients, to address the need for a reliable and sensitive technique or combination of tests to the detection of subclinical infection, with low numbers of bacilli present and no evident symptoms of clinical activity, in potential high risk groups in contact with leprosy patients.
Materials and methods

PATIENTS AND CONTACTS

Sixty leprosy patients attending the Sanatorium of Fontilles (Spain) were included in this study. All procedures were in accordance with the standards and guidelines of the Fontilles Ethical Committee. Fifty multibacillary patients were included, of whom 30 had completed multidrug therapy (MDT), 20 showed a positive bacteriological index (BI) and were still on MDT treatment, and 10 patients were classified as paucibacillary (PB) and had completed 6 months MDT. The MDT (OMS) used was a combination of 600 mg/monthly rifampin, 100 mg/daily clofazimine and 100 mg/daily dapsone, until the BI became negative, a minimum of 2 years; PB patients received a combination of 600 mg/monthly rifampin and 100 mg dapsone daily for 6 months.

The 10 PB cases included in this study were all diagnosed 4–15 years before the introduction of MDT by WHO, and treated over many years with 100 mg of dapsone monotherapy. They were all treated for 6 months with WHO MDT and 8 years after completing treatment, presented no signs of clinical activity. These patients were diagnosed on clinical criteria and AFB could not be detected by staining of skin smears or histopathological sections and microscopic examination. The histological diagnosis was based on ‘epithelioid granulomas resembling tuberculoid leprosy’. None of the patients presented reversal reactions or other symptoms of clinical reactivation.

Patients were grouped clinically and histopathologically by the Ridley–Jopling classification and for the purpose of this study into the WHO 1987 classification, based on the criteria that all patients showing smear positivity should be classified as having multibacillary (MB) leprosy for the purpose of MDT programmes and the negative group with no detectable AFB as paucibacillary (PB) leprosy. Their ages were between 27 and 90 years; 23 females and 37 males. Four non-leprosy patients and 50 sera obtained from the individuals control were also included as controls of PCR-techniques and serological methods respectively.

Forty-three individuals with more than 8 years of contact with leprosy patients were also included in this study and divided into two groups: 12 household contacts (HC) defined as persons sleeping during the night under the same roof and 31 occupational contacts (OC), including hospital staff and other workers of the Sanatorium.

CLINICAL SAMPLES

Slit-skin smears, slit-skin swabs and biopsies were taken from an active and old skin lesion of the leprosy patients and controls all at exactly the same site, together with serum samples, smears and swabs from ear lobe and nasal swabs.

A combination of less invasive procedures (nasal swabs, serum samples and intradermal injection of lepromin A were obtained from or carried out on the group of contacts.

Serum samples

Blood samples were collected by venipuncture from all patients and contacts and, following centrifugation, serum was separated and kept frozen at −40°C until processed. Forty serum samples were also collected from individuals attending the local General Hospital and with no known history of mycobacterial diseases, as controls for the serological assays, together
with 10 samples of serum from active tuberculosis patients, diagnosed clinically and
bacteriologically to check possible cross-reactions with the antigens used.

**Slit-skin smears, biopsies and swabs**

Slit-skin smears and excision biopsies were taken from the same site of old or active skin
lesions. From the same cuts for the smears and excision produced by biopsy, swabs were
taken, so that every patient had from exactly the same site: a slit-skin smear, slit skin swab
(pre-biopsy swab), biopsy and swab from the biopsy excision (post-biopsy swab). The study
was completed with slit-skin smears and swabs from ear lobes and a nasal mucosa scraping
and swab from each patient.

Nasal mucosa swabs were also obtained from contacts. These swabs were taken by
introducing a cotton-tipped swab (Medical Wire and Equipment Co.) 2–3 cm into each nostril
and rubbing the swab several times over the inferior lateral conchae.

Slit-skin smears were prepared on microscopic slides and stained by the classical Ziehl–
Neelsen method. Biopsies were divided into two parts: one half was fixed in 5% (V/V)
buffered neutral formalin and then dehydrated in a graded series of ethanol and embedded in
paraffin. Sections were stained by the Fite–Faraco method. The other half was frozen in
liquid nitrogen for PCR studies. All swabs were frozen at −20°C pending their analysis.

**LEPROMIN TESTING**

Lepromin A, kindly provided by the GWL Hansen’s Disease Center, Louisiana State
University, was injected intradermally at the dose of 0.1 ml and concentration of 4-6×10^7 bacilli/ml. The Mitsuda reaction was read 21 days after injection as the diameter
of the nodule in mm.

**SEROLOGICAL ASSAYS**

**D-BSA**

IgM anti-D-BSA antibodies was measured by an indirect ELISA according to the method
described by Fujiwara et al.\(^\text{15}\) Briefly, the antigen D-BSA was used at the concentration of
2 μg/ml. The serum was prediluted at 1/300 in phosphate buffered saline (PBS) and were
incubated with goat anti-human IgM antisera peroxidase conjugated diluted 1/2000 and
then 100 μl of H₂O₂-O-phenylenediamine was added. The reaction was stopped at 20 min by
the addition of 50 μl of 2.5 N sulphuric acid and the trays read with a Dynatech Automated
Reader. Samples with an absorbance at 492 nm of >0-100 OD (the mean absorbance plus
three standard deviations of the healthy control subjects) were considered positive. Known
positive controls of pooled multibacillary patients serum and negative controls were included.

**PGL-1**

IgM anti-phenolic glycolipid (PGL-1) antibodies were measured by an indirect ELISA
according to the method described by Cho et al.\(^\text{16}\) with minor modifications. The antigen was
used at 5 μg/ml and serum samples were diluted 1/300 in PBS with 5% BSA. Goat anti-
human immunoglobulin M-peroxidase reagent was used at 1/1000 dilution and after 1 h
incubation, 100 µl H2O2-O-phenylenediamine substrate-dye reagent was added. The reaction was stopped at 20 min with 50 µl of 2.5 N sulphuric acid and read as for D-BSA at 492 nm with absorbance values >0.160 OD considered positive.

The cut-off values considered seropositive and determined in the control group are 0.160 for PGL-1 and 0.100 for D-BSA.

**PCR METHODS**

**DNA extraction**

The frozen sections of skin biopsies specimens were incised to small pieces (5 µm thick) with sterile scissors and incubated with 50 µl of lysis buffer (Tris-HCl 100 mM pH 8.5, Tween 20% 0-05% and proteinase K 60 µg/ml) for 18 h at 60°C and finally 15 min at 97°C to inactivate proteinase K.6 Paraffin oil (40 µl) was layered on top to prevent evaporation. Swabs were treated by the method described by Williams et al.17 with slight modifications. Each swab was cut above the cotton wool and transferred to a 0.5 ml Sarstedt vial containing 200 µl of 3% Triton-X 100 and incubated at 97°C for 10 min. All samples were then centrifuged at 13,000 rpm for 5 min and transferred to an Eppendorf tube. Aliquots of 5 µl of lysates from the biopsy and swab extract were used to posterior DNA-amplification methodology.

*M. leprae* DNA control was kindly provided by Dr P. Brennan, Department of Microbiology, College of Veterinary Medicine and Biomedical Science, Colorado (USA) and obtained from experimentally *M. leprae* infected armadillos and consists of a fair amount of sheared DNA as well as a high molecular weight DNA and was used as a positive control in all PCR experiments. Sterile distilled water was used as a negative control. The purified *M. leprae* DNA (15 µg/ml) was diluted in 10 ml sterile distilled water to a final concentration of 1.5 µg/ml. Final working concentration was 500 pg/ml was and 5 µl of the dilution sample was then added to the PCR mixture.

**PCR primers selected**

The primers used for amplification were designed by Hartskeerl et al.7 and were selected on the basis of the nucleotide sequence of the gene encoding the 36-kDa antigen of *M. leprae*. The primers used were S13 and S62. The sequences of the primers which amplify a 531-bp fragment of the *M. leprae* DNA sequence were S13 (5’-CTCCACCTGAGCGGCGAT-3’) and S62 (5’-GACTAGCCTGCCAAATCG-3’). The detection limit of the primers in this assay was between 10 and 100 bacilli, being the highest dilution of chromosomal DNA *M. leprae* (62.5 femtograms) showing amplification.

**DNA amplification**

DNA extracted (5 µl) to biopsies or swabs was added to the cocktail of amplification to a total amount of 50 µl, containing a reaction mixture of 1 µM of each primer (S13 and S62),7 0.2 mM of each nucleotide, 2.5 IU of Taq DNA polymerase and 5 µl of buffer I 500 mM KCl, 100 mM Tris-HCl pH 8.3 and 15 mM MgCl2 for biopsies and 5 µl of buffer V 500 mM KCl, 100 mM Tris-HCl pH 8.8, 15 mM MgCl2 and 1% Triton X-100 for swabs. The amplification was performed in a Perkin-Elmer automated Thermal Cycler with an initial denaturation step
(95°C/3 min) followed by 45 cycles (denaturation 94°C/2 min, annealing 55°C/2 min and elongation 72°C/3 min) and a final extension step (72°C/12 min).

A 531 bp amplification product was obtained by electrophoresis on 2% agarose gel. The DNA was stained with ethidium bromide and visualized on a 302 nm, ultraviolet transilluminator (Figure 1).

In order to test for the presence of PCR inhibitors, PCR negative specimens were re-tested after adding 5 μl of the highest dilution of chromosomal DNA showing amplification to the amplification mixture.

Results

A comparison of the results with the two groups of methods (conventional and PCR) for the detection of \textit{M. leprae} in the different clinical specimens is shown in Table 1. No false positive results were detected by either technique. Paucibacillary patients (Ridley–Jopling classification\textsuperscript{18}) were negative for all tests.

In the MB BI+ group, the histopathological sections of new or old lesions stained by Fite–Faraco detected AFB in 19/20 (95%) of cases (the AFB negative case with the histopathological sections proved AFB positive by slit-skim and ear lobe smears), followed by slit-skin smear (75%), ear lobe (55%) and nasal swab (40%).

The techniques in the PCR group detected \textit{M. leprae} DNA at higher rates than the conventional methods at all sites tested. There were significant differences in the detection rates of \textit{M. leprae} DNA: nasal mucosal staining (40%) versus PCR-nose swab (85%) \textit{(P}<0.01) and ear lobe smear (55%) versus PCR-ear lobe swab (85%) \textit{(P}<0.01) (Table 1).

Biopsy-PCR and post-biopsy swab detected \textit{M. leprae} DNA in all 20 cases (100%) and the results were not significantly different to the detection rate (95%) of conventional histopathology (19/20).

In the MB BI− group, the conventional methods (slit-skin smear and histopathological sections) detected one case of clinical relapse in a patient who had completed MDT 3 years before (1/30). All the remaining patients in this group were AFB negative at all sites, while
with the PCR techniques, three patients were *M. leprae* DNA positive by PCR-biopsy (3/30) and 6/30 by the PCR-post-biopsy swab (including the three PCR-biopsy positives). Both techniques detected the clinically relapsed patient of the previous group.

In the PB group, no AFB or 531 bp bands on agarose gel after PCR were detected. The seropositivity rate comparison of the two antigens (PGL-1 and D-BSA) for the different leprosy patients groups is shown in Table 2. There was one false positive (2.5%) for each antigen in the control group. No seropositivity to any of the two antigens was detected in the tuberculosis group.

When combining the seropositivities of the different assays, the overall percentage of persons being seropositive to any of the two tests in each group was the same as the percentage detected by IgM-D-BSA, alone except in the control group.

In the MB+ group, seroprevalence rates to PLG-1 and D-BSA were 60% (12/20) and 85% (17/20), respectively, and statistically significant (*P* < 0.05). D-BSA did not detect an anti-IgM humoral response in three patients with positive BI detected by conventional methods and PCR techniques (two with BI = 2 and one with BI = 1). In the MB BI negative group, the different seropositivity rates (10% GLP-1 and 13.3% D-BSA) were not statistically significant. Only one PB patient had a positive serological response to D-BSA. Three of the 43 contacts (6.9%) had high anti-IgM antibody levels to PGL-1 and/or D-BSA compared to 1/40 (2.5%) in the control group.

Table 1. Comparison of sensitivity between conventional and molecular biology (PCR) at several different sites, examined in multibacillary (MB; *n* = 20) positive and negative (*n* = 30) and paucibacillary (PB; *n* = 10) patients and non-leprosy controls (*n* = 4)

<table>
<thead>
<tr>
<th>Methods</th>
<th>MB (+)</th>
<th>MB (−)</th>
<th>PB</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal swab</td>
<td>40% (8)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ear lobe</td>
<td>55% (11)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slit-skin smear</td>
<td>75% (15)</td>
<td>3-3% (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Biopsy-histopathology</td>
<td>95% (19)</td>
<td>3-3% (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal swab</td>
<td>65% (13)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ear lobe swab</td>
<td>85% (17)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slit-skin smear swab</td>
<td>80% (16)</td>
<td>3-3% (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCR-biopsy-histopathology</td>
<td>100% (20)</td>
<td>10% (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Post-biopsy swab</td>
<td>100% (20)</td>
<td>20% (6)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Seropositivity rate of PGL-1 and D-BSA for multibacillary BI positive (*n* = 20) and negative patients (*n* = 30), paucibacillary (PB; *n* = 10) and tuberculosis patients (TBC; *n* = 10), household (HC; *n* = 12) and occupational contacts (OC; *n* = 31) and healthy controls (*n* = 40)

<table>
<thead>
<tr>
<th>Test</th>
<th>MB (+)</th>
<th>MB (−)</th>
<th>PB</th>
<th>TBC</th>
<th>HC</th>
<th>OC</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-PGL-1</td>
<td>60% (12)*</td>
<td>10% (3)</td>
<td>0</td>
<td>0</td>
<td>8-3% (1)</td>
<td>3-2% (1)</td>
<td>2.5% (1)</td>
</tr>
<tr>
<td>IgM-D-BSA</td>
<td>85% (17)</td>
<td>13.3% (4)</td>
<td>10% (1)</td>
<td>0</td>
<td>16-6% (2)</td>
<td>3-2% (1)</td>
<td>2.5% (1)</td>
</tr>
<tr>
<td>Both</td>
<td>85% (17)</td>
<td>13.3% (4)</td>
<td>10% (1)</td>
<td>0</td>
<td>16-6% (2)</td>
<td>3-2% (1)</td>
<td>5% (2)</td>
</tr>
</tbody>
</table>

* Seropositivity is shown as percentage, with the number of positives in parentheses.

* Seropositivity in any of the two tests.
Comparison of serological responses to D-BSA and PGL-1, PCR techniques and conventional methods on the different leprosy groups included in the study, and length of MDT treatment (more or less than 3 months) in MB+ leprosy patients is shown in Table 3. The results presents the overall detection rates of all the different samples and methods used in this study on the multibacillary BI+ patients, reflecting the importance of the interval or length of treatment on the sensitivity of the tests employed. Two subgroups of MB+ patients can be differentiated according to the length of treatment and bacterial load (BI). In the subgroup of patients with <3 months of MDT and an average BI of 3, all the techniques give detection rates between 90 and 100%. The two serological markers detect the same 9/10 patients with high average optical densities of 0.790 for PGL-1 and 0.868 for D-BSA. In the other subgroup of individuals with between 3 and 12 months of MDT and average BI of 1-3, D-BSA still detected a humoral response in 8/10 with an average OD of 0.274, while PGL-1 seropositivity dropped to 30% (3/10 and 0.146 average OD).

With regard to serological data and length of treatment with MDT, compared with conventional and molecular methods, obtained in the MB BI+ group, no relation could be established between age or sex. Although the number of cases studied was small and the treatment time periods rather large, a trend can still be observed in which the detection rates of some of the tests, especially in the conventional method group, and the humoral responses to PGL-1 decreased sequentially with time after drug treatment. During the first 3 months of MDT, with a high bacterial load, all the different techniques gave high detection rates in all three groups of methods (90–100%), with seropositivity rates for both antigens in this stage at 90%. In the other subgroup with a period of treatment between 3 and 12 months and an average group BI of 1-3, there is a lack of sensitivity after 3 months when MDT has killed 99% of all viable bacilli and total AFB are small in number; only biopsy-histopathology detected 90% (9/10) of the cases.

<table>
<thead>
<tr>
<th>Table 3. Relationship between serological responses to D-BSA and PGL-1, conventional methods and PCR techniques and length of treatment (MDT) in MB (+) leprosy patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length of treatment (MDT)</strong></td>
</tr>
<tr>
<td>&lt;3 months</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td><strong>Bacteriological index</strong></td>
</tr>
<tr>
<td><strong>Serological methods</strong></td>
</tr>
<tr>
<td>PGL-1</td>
</tr>
<tr>
<td>D-BSA</td>
</tr>
<tr>
<td><strong>Conventional methods</strong></td>
</tr>
<tr>
<td>Nose swab</td>
</tr>
<tr>
<td>Ear lobe</td>
</tr>
<tr>
<td>Slit-skin smears</td>
</tr>
<tr>
<td>Biopsy-histopathology</td>
</tr>
<tr>
<td><strong>PCR methods</strong></td>
</tr>
<tr>
<td>Nose swab</td>
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<tr>
<td>Ear lobe swab</td>
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<tr>
<td>Slit-skin swab</td>
</tr>
<tr>
<td>PCR-histopathology</td>
</tr>
<tr>
<td>Post-biopsy swab</td>
</tr>
</tbody>
</table>

*Average antibody level of the group.*
Table 4. Combination of tests (lepromin A, ELISA antibody using PGL-1 and D-BSA antibodies and DNA M. leprae nasal carriage by PCR) on household and occupational contacts of leprosy patients

<table>
<thead>
<tr>
<th>Contacts group</th>
<th>Number</th>
<th>Lepromin A</th>
<th>Nasal-swab</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Household</td>
<td>1*</td>
<td>N</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>N</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Occupational</td>
<td>1*</td>
<td>N</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

NN: negative, P: positive results.
* Two cases NPP detected.

In MB BI— patients, the PCR post-biopsy swab detected M. leprae DNA in 6/30 cases in this group. One of these patients was also positive by other techniques (conventional histopathology, PCR-biopsy and serology PGL1/D-BSA) and another by PCR-biopsy and GLP-1/D-BSA; both were clinically diagnosed as type 2 (ENL) and relapse. The remaining PCR post-biopsy swab and PCR-biopsy positive patients, together with two cases that presented high antibody levels to D-BSA, showed no clinical symptoms or activity and are currently under follow-up study for possible early detection of relapse.

In the contact group, 25 of the 43 contacts were lepromin A negative, including the two individuals positive for the previous tests. The combination of tests employed to study the transmission of the disease and detection of a potential high-risk group in contact with leprosy patients are presented in Table 4. The combination NPP (lepromin A negative; nasal swab M. leprae DNA positive and PGL-1 or/D-BSA IgM antibody positive detected in two cases (one household and one occupational) was considered of high risk contact individual, together with one case NNP.

Discussion

Leprosy diagnosis can be established in most cases by the traditional methods of clinical examination, skin smears and histopathology. The results in our study indicate that histological examination after staining of new or old lesions is very sensitive for the detection of AFB bacilli, especially in the multibacillary form. These results confirm that the microscopic examination of histopathological sections is the conventional method most indicated for establishing the definitive diagnosis of leprosy.19 Skin slit smears from several sites (skin lesion and ear lobes), together with nasal mucosa stained swabs, also gave high rates of AFB detection and are less invasive and demanding for monitoring the patients progress during treatment. The PCR techniques present 100% detection rates with PCR-biopsy and PCR- post biopsy swab in this MB BI+ group.

Although other studies have confirmed a good correlation (r = 0.926) between PGL-1 and D-BSA in serological study leprosy, showing similar sensitivity and specificity,20 we also evaluated these antigens on our population of patients. D-BSA (85% positive) showed a higher seroprevalence compared with PGL-1 (60%) but did not detect anti-IgM antibodies in
three cases positive by other techniques. A possible explanation may be that some patients respond predominantly to *M. leprae* antigens with IgG antibody formation21 or the lack of circulating antigens during certain periods of treatment with MDT.22 The difference in seropositivity rates (Table 2) in multibacillary BI positive patients (*n* = 20) between IgM-PGL-1 and IgM-D-BSA antibodies (60 versus 85%) is due to the decrease in the serological response detected by PGL-1 in the average 1-3 BI group (30%), and these results suggest that D-BSA in our study is a better serological marker for monitoring the reduction of the bacterial load during course of chemotherapeutic treatment.23

The higher sensitivity of the PCR techniques of the gene encoding the 36-kDa antigen of *M. leprae*, which detected 10^2* bacilli in this study, was still theoretically more sensitive than other methods such as microscopy for the direct detection of *M. leprae*, with 100% positives on biopsy and post-biopsy swab samples. This result makes this technique a useful tool in the follow-up of these patients to assess bacterial load reduction during the course of drug treatment, or of relapsed cases in which the number of viable bacilli has increased again after an initial decrease during the course of treatment.

In the MB BI− group, several patients in whom microscopic examination did not reveal any AFB gave positive results with PCR. This is in agreement with results obtained by other authors.24 The PCR-biopsy and PCR-post biopsy swab detected *M. leprae* DNA on three and six out of 30 samples, respectively. Two of these cases were confirmed by other techniques and clinically diagnosed as reaction type 2 (ENL) and relapse. The detection of patients still positive by PCR for *M. leprae* specific DNA is worth taking into account, since they had all completed a minimum 2 years treatment with MDT. Although it is possible that the amplified DNA is from dead bacilli, it would be surprising if such large fragments of DNA could survive in living host tissues for long after the bacilli were killed.25

Although a relationship between positive PCR signals and potentially viable *M. leprae* has been suggested by several authors and could be very significant in this type of patient with small numbers of bacilli,10,26 well controlled studies are needed to determine the possible relation between the *M. leprae* DNA amplification obtained and viability. Mouse inoculation techniques should be employed in these cases to evaluate if relapse is caused by drug-sensitive persisters or the presence of drug resistance27 and those patients potentially at risk of relapse should be carefully followed up. 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. However, the persistence of *M. leprae* DNA positivity in some multibacillary patients for a long time even after completion of treatment without apparently causing any damage to the host shows that it is not possible to consider PCR positivity as a diagnostic test for relapse. Further investigations are still needed to draw final conclusions on the clinical significance of these results.

We tried to extend the diagnosis of leprosy in this study by the investigation of different types of clinical samples, and a PCR post-biopsy swab sample was evaluated, although no information could be found in the literature concerning this technique. In the MB+ group, together with PCR-biopsy, this was the only technique that detected positivity in all cases and continued presenting the highest detection rate of positivity in the MB− group, confirming the results obtained in some of these patients by other techniques. The remaining three cases were only post-PCR biopsy positive, and biopsy PCR-inhibition was proved absent by retesting with a DNA control. Although the number of samples is small, a possible explanation could be that the swab was taken on the skin, possibly infected by AFB that was altered and remained in the surgical excision, since the swab was taken immediately after the biopsy. The
technique is a simple and rapid extraction procedure (no long 16 h period of incubation required with biopsy samples), and preliminary results are most encouraging for its potential, together with other PCR techniques, to improve the diagnosis of leprosy cases with low numbers of bacteria, although it still requires further evaluation.

In the PB group, no positivity was detected by conventional or PCR methods (only one patient had a serological response to D-BSA) in any of the patients studied. These results are disappointing, since it is these patients where AFB bacilli are rare or absent and conventional methods fail to detect their presence that new and more sensitive techniques are needed. However, only 10 PB treated several years ago were tested, and it is not surprising that they were negative on all these techniques.28

Several studies have reported promising results with PCR amplification techniques in PB patients for diagnosis prior to treatment. One study found 20/27 (74%) biopsies positive using primers that amplify a 360 bp specific fragment of M. leprae DNA of the gene that encodes the 18-kDa protein of M. leprae. This result can be compared with detection of AFB in only 1/27 of the same biopsy samples using conventional histopathology;29 8/21 biopsies (38%) using our same protocol detected M. leprae DNA in PB patients treated with sub-optimal doses of 50 mg daily of dapsone monotherapy.30 Other studies reveal a lack of correlation between humoral responses (anti-IgM) to PGL-1 and M. leprae DNA specific positivity by PCR in this group of patients.30

Through the application of PCR, it is now possible to detect nasal carriage of M. leprae DNA directly and specifically.31,32 In this study, two individuals had nasal carriage of M. leprae DNA detected by PCR (one household contact of a MB patient and one occupational contact). Since nasal carriage was not evaluated in a non-contact group, no comparison between contacts and noncontacts can be established.

Several studies have reported no significant differences in PCR positivity rates between contact and non-contact populations, indicating that the general population in areas where leprosy is endemic face similar risks of exposure to M. leprae,13,33 results also obtained in seroepidemiological studies,34 where the prevalence of antibody positivity to specific serological antigenic marker of M. leprae is considered a symptom of systemic involvement due to continued exposure to the organism in leprosy endemic countries. Our three cases form a subgroup with over 20 years of exposure and contact with non-treated multibacillary patients that is considered the main source of infection in our non-endemic country35 or the potential role of nasal carriage of M. leprae in healthy asymptomatic people as transient carriers and maintenance of infection reservoirs. The seroprevalence in our contact group (6-95%) compared with 2-5% in the non-contact group was nearly significant. The numbers of individuals in the household and occupational groups were too small to be considered separately.

Although some studies have found no correlation between M. leprae DNA nasal carriage and serology to specific M. leprae antigens,36 two cases in our study gave positive results in both tests, suggesting that nasal immunity (the nasal mucosa is probably the main port of entry), considered an important defence against further dissemination of the bacilli,37 has failed to detain a transient carrier state without colonization resulting in infection and that serological responses to M. leprae specific antigens are more prevalent in the MB form of leprosy, which is the predominant type of disease in this country.

The prognostic value of the lepromin test has been demonstrated, showing that the risk of lepromatous leprosy is greater in persistently lepromin negative individuals.38 Their potential risk for developing disease is awaited, and implies repeated serial clinical and laboratory
testing to evaluate the potential of this combination for predicting development of disease, although similar situations have lead to the administration of chemoprophylaxis. In England, two long time lepromin A negative and PGL-1 seropositive household contacts of a non-diagnosed MB patient were treated during 6 months with rifampin 30 and household contacts of MB cases in high endemic areas were administered a single dose of ROM (600 mg rifampin, 400 mg ofloxacin and 100 mg minocycline) 40 to prevent clinical disease and eliminate the chain of possible transmission. In our leprosy contact study, the combination of conventional and molecular methods showed three cases considered to be high risk contacts, two NPP cases, one household and one occupational, (lepromin A negative; nasal swab M. leprae DNA positive and PGL-1 or/and D-BSA IgM antibody positive), together with one NNP case. These patients were considered as a the high-risk contact group, and should be included in the control measures and chemoprophylaxis.

References

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