Polymerease Chain Reaction for Detection of *Mycobacterium leprae* in Nasal Swab Specimens

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The polymerase chain reaction based on the selective amplification of a 531-bp fragment of the gene encoding the proline-rich antigen of *Mycobacterium leprae* was applied to nasal swab specimens from leprosy patients, occupational contacts, and endemic and nonendemic controls. To prevent false-positive amplification, we used dUTP and uracil-DNA-glycosylase in all polymerase chain reactions. False-negative reactions were detected by using a 531-bp modified template as an internal control. Amplification products were found in 55% of untreated patients, in 19% of the occupational contacts, in 12% of endemic controls, and in none of the nonendemic controls. This study strongly suggests that not only leprosy patients but also healthy persons may carry *M. leprae*. We concluded that polymerase chain reaction is a reliable method to detect *M. leprae* in nasal specimens. The method holds promise for studying the spread and transmission of *M. leprae* within a population.

Little is known about the scope and importance of subclinical infection with *Mycobacterium leprae*, the causative agent of leprosy. Measurement of the proportion of subclinical infections in a given population will enhance our knowledge of the spread and transmission of *M. leprae* and associated risk factors. In addition, an ability to recognize infection with *M. leprae* will improve our understanding of the natural history of leprosy. This might ultimately have important implications for leprosy control, particularly if it would permit definition of targets for prophylactic and therapeutic strategies.

Studies employing immunological tests for the identification of subclinical infection have been inconclusive (5, 8, 19). The possibility exists that individuals are seropositive because of a past infection. In contrast, they may well be colonized with *M. leprae* before the immune response is triggered. Detection of *M. leprae* bacilli has been hampered by our inability to grow *M. leprae* in vitro. Existing laboratory methods lack both sensitivity and specificity to detect small numbers of bacilli.

Our previously described polymerase chain reaction (PCR) was shown to be applicable to the specific detection of small numbers of *M. leprae* bacilli in biological specimens (4, 9). Because it is recognized that in leprosy the nasal cavity is involved in carriage and shedding of *M. leprae* (2, 3) and because the nose is considered to be one of the likely ports of entry of the bacilli (11, 12, 16, 17), we further developed this PCR to make it applicable to nasal swab specimens. To minimize false-negative results due to inhibitory components from nasal mucosa, we improved the reaction conditions by the addition of dimethyl sulfoxide (DMSO), which has been reported to alter the amplification efficiency (1). Furthermore, we constructed a 531-bp modified template to serve as an internal control. Samples that did not show amplification of *M. leprae* target DNA were run jointly with this modified template to monitor whether the negative result could have been caused by inhibitory components from the specimen. In this way, false-negative results were excluded. The reliability of the procedure was further optimized by the use of dUTP and uracil-DNA-glycosylase (UDG) for the prevention of false-positive results due to carryover of previously amplified DNA (14).

Using this optimized protocol, we investigated nasal swab specimens from clinically diagnosed leprosy patients to establish to what extent the PCR method could detect carriage among patients. On the basis of the hypothesis that *M. leprae* is present in the nasal mucosa of not only leprosy patients but also healthy persons, we also investigated nasal swab specimens from persons living in areas where leprosy is endemic and in those where it is not endemic, the former consisting of occupational contacts and persons not having direct contact with leprosy (endemic controls).

**MATERIALS AND METHODS**

Collection of nasal swab specimens. Nasal swabs (Medical Wire and Equipment Co.) were used to collect nasal specimens from 25 healthy Dutch volunteers and from 81 persons in the Philippines. Of these, 25 were leprosy patients and 56 were staff members of a hospital. Of the leprosy patients, 20 were untreated and 5 were treated, multibacillary (MB) patients. The 56 hospital staff members made up two groups: one group (*n = 31*) was considered occupational contacts, working with leprosy patients daily; the second group (*n = 25*) had no direct involvement with leprosy patients and members were considered endemic controls. The specimens were collected by gently rubbing the swab over the nasal septum several times. The samples collected in the Philippines were coded, and PCR was performed without prior knowledge of the classification of the sample. The classification of leprosy was based on the enumeration of acid-fast bacilli in skin biopsy specimens of all patients by means of the bacterial index (BI) of the granuloma on

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sections stained by the modified Fite method (18). MB patients were defined as having a BI of >0, and patients who had a BI of 0 were defined as paucibacillary. From four untreated MB patients two extra nasal swab specimens were collected simultaneously with the one used for the PCR study. One was smeared on a glass slide for determination of the BI, while the other was used in an enzyme-linked immunosorbent assay (ELISA) for detection of the M. leprae-specific phenolic glycolipid I antigen.

All swabs were stored at −20°C pending analysis.

**Controls.** *M. leprae* was isolated from liver tissue of an experimentally infected armadillo, using the protocol recommended by the World Health Organization (20). Enumeration of the acid-fast bacilli was done on Ziehl-Neelsen-stained smears of the final preparation.

Chromosomal *M. leprae* DNA was kindly supplied by M. J. Colston as part of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. This DNA served as a positive control in all PCR experiments.

From the swabs collected from the Dutch volunteers, 11 were used as nonendemic controls and 14 were spiked with *M. leprae*, in amounts ranging from 10⁶ to 10 per swab, to monitor the optimized conditions throughout the procedure.

**Preparation of specimens.** Each swab was cut above the cotton wool and transferred to a 0.5-mL Sarstedt vial containing 100 μL of lysis buffer covered with 40 μL of paraffin liquid. The composition of the lysis buffer was the same as described previously (4), except the proteinase K was raised to a final concentration of 1 mg/mL. Incubation of the samples was carried out as described previously (4), after which the vials were centrifuged for 10 s at 10,000 × g, with the cotton wool remaining in the lysis buffer. Two microliters of the lysate was subsequently submitted to PCR.

*M. leprae* suspensions were treated the same way, except that the centrifugation step was omitted.

**PCR.** To determine the presence of *M. leprae* in specimens, PCR runs were performed in the following order. To perform the first PCR (PCR-A), 2 μL of the lysate was added to a 48-μL reaction mixture containing 10 mM Tris-HCl (pH 9.6), 50 mM NaCl, 0.01% gelatin, 5 mM MgSO₄, 100 ng each of primers S13 and S62, 10% DMSO, 1 mM each dATP, dCTP, dGTP (Pharmacia), and dUTP (Boehringer Mannheim), 0.5 U of UDG (Bethesda Research Laboratories), and 2.5 U of thermostable Taq DNA polymerase (Perkin-Elmer Cetus). For UDG activation, samples were incubated at 25°C for 10 min, and for degradation of fragments, samples were incubated at 95°C for 10 min. After this, 37 cycles were performed, each consisting of the following steps: 2 min of denaturation at 94°C, 2 min of annealing at 60°C, and 3 min of elongation at 72°C.

In each run, positive controls of 375 pg, 75 pg, 15 pg, 3 pg, 600 fg, and 120 fg of chromosomal *M. leprae* DNA were included, as well as six negative controls without target DNA (i.e., lysis buffer). Samples were subsequently analyzed for the presence of a 531-bp amplification product by gel electrophoresis in a 2% (wt/vol) agarose gel.

Samples in which a 531-bp fragment was not detected were submitted to a second PCR (PCR-B) to determine whether the sample contained inhibiting components that could have caused the negative result in PCR-A. In the second PCR, 15 copies of a 531-bp modified template (see below) were added to a reaction mixture otherwise having the same composition as the one used in PCR-A. Two microliters of the lysed sample was run jointly with the modified template, using the same run profile as for PCR-A. Samples were tested on a 2% (wt/vol) agarose gel. When samples were found to inhibit amplification of the modified template, 50 μL of the lysate was subsequently submitted to purification, using the Isogene Kit (Perkin-Elmer Cetus) in accordance with the manufacturer’s instructions. The DNA-binding matrix was eluted with 50 μL of distilled water; 5 μL of this eluate was run in PCR, with and without the modified template. The PCR run with the modified template was to monitor the effect of the purification.

To confirm that each 531-bp fragment represented an amplification product from the *pra* gene region, samples were analyzed by hybridization. Each sample was transferred to a Duralose UV membrane (Stratagene), using a Vacuum Blotting System (Pharmacia). Blots were hybridized by using the 1.0-kb EcoRI fragment containing the *pra* gene of *M. leprae* as a DNA probe, as described previously (9).

A specimen was considered positive when it revealed, with or without purification of DNA, a 531-bp fragment by both agarose gel electrophoresis and subsequent hybridization.

A sample was considered negative when it did not show amplification in PCR-A and did not inhibit the amplification of the modified template in PCR-B.

**Construction of the modified template.** Two primers were selected to perform site-directed mutagenesis on the 531-bp fragment of the *pra* gene of *M. leprae* to remove the HaeIII restriction site (GGCG) located at positions 864 to 867. The mutagenesis was chosen such that digestion with restriction enzyme HaeIII made it possible to differentiate between the nonmodified and the modified 531-bp fragments, with the former showing 279-, 213-, and 39-bp fragments and the latter showing 492- and 39-bp fragments after digestion. The selected primers were complementary and homologous in sequence to position 5' 857–875 3' of the *pra* gene, except for a single-base mismatch at position 865, in which primer 1 dGTP was replaced by dTTP and primer 2 dCTP was replaced by dATP.

**Left-site mutagenesis (PCR-I).** This was achieved by amplifying 1 ng of chromosomal *M. leprae* DNA, using outside primer S13 and primer 2. For right-site mutagenesis (PCR-II), outside primer S62 and primer 1 were employed. The resulting PCR products, a 262-bp fragment from PCR-I, and a 288-bp fragment from PCR-II, were mixed in equal volumes and diluted 1:10,000 in distilled water. Of this dilution, 5 μL was submitted to PCR-III, using the outside primers S13 and S62. The resulting 531-bp fragment was purified from a 2% (wt/vol) agarose gel, using the Geneclean Kit (Bio 101 Inc.). Digestion with restriction enzyme HaeIII was performed to confirm that the 531-bp fragment was indeed modified.

Fifty nanograms of the Gene Clean-purified, modified fragment was used for blunt-end cloning (15), using pUC-19 as a vector (Bethesda Research Laboratories) and E. coli DH5-α (Bethesda Research Laboratories) as competent cells.

Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (10). The concentration, as determined by optical density at 260 nm, was 7.5 ng/ml, which equals 3 × 10¹⁴ copies of inserted modified 531-bp fragment per μL. The amount of the modified template to be used in PCR-B was determined by running jointly known amounts of *M. leprae* DNA, i.e., 375 pg, 75 pg, 15 pg, 3 pg, and 600 fg, each with the following numbers of copies of the modified template: 1.5
A nasal swab specimen was considered positive when the optical density was at least three times that of the monoclonal antibody incubated on noncoated wells.

RESULTS

Optimization of the PCR procedure: spiked swabs. The yield of amplified DNA obtained with the spiked swabs was compared with the yield obtained by the same amounts of bacilli processed from a swab not containing a nasal specimen. Reduction of the lysate volume added to the PCR reaction mixture in combination with the addition of DMSO proved to be essential. Lysate volumes of 25, 15, 10, 5, and 2 μl were tried. When 2 μl of the lysate was added to the reaction mixture containing 10% DMSO, an amplification product was observed in all spiked samples (Fig. 1). No amplification products were observed when a lysate volume of >5 μl was added to the PCR reaction mixture (data not shown).

Prevention of false-positive results. The effectiveness of the use of dUTP and UDG is shown in Fig. 2. When two subsequent PCR runs were performed, no amplification was observed in the second run, indicating that PCR based on incorporation with dUTP results in amplicons that are completely degraded after UDG incubation.

Modified template. To monitor whether a joint run of M. leprae DNA (i.e., wild-type DNA) and the modified template would affect the amplification of either of the two types of DNA, we amplified various numbers of copies of the modified template together with standard amounts of M. leprae DNA.

The results are shown in Fig. 3. M. leprae DNA showed the same degree of amplification when run jointly with the different numbers of copies of the modified template as the same amounts of DNA run separately. Furthermore, M. leprae DNA did not inhibit the amplification of the modified template in the various numbers of copies added. The 39-bp fragment, present in both restriction patterns, could not be visualized on the gel because of the small quantity present. However, for the use of the modified template with nasal swab specimens, we had to perform two separate PCR runs. Preliminary experiments showed that the amount of M. leprae in those specimens was usually too low to make the restriction pattern of the resulting amount of amplified DNA visible on the gel. To check that the amplification of target DNA was not inhibited in the first PCR, the modified template was added as an internal control in the second PCR.

Application to nasal swab specimens. The percentages of positive results in the PCR on the nasal specimens from all groups are shown in Fig. 4. Amplifications were observed in 11 (55%) of 20 untreated MB patients. Among these were three of the four patients who had a positive BI on the nasal specimen. No amplification products were observed in the specimens from four MB patients under treatment. The possible presence of M. leprae in the specimen from one more treated patient could not be determined, because the amplification of the modified template was inhibited, even after purification.

Amplification products were found in 6 (19%) of the 31 occupational contacts. From 11 persons of this group a second specimen was collected 1.5 years after the first one. Two of these showed a positive result in both the first and second samples, and seven showed a negative result in both samples, while two showed a positive result in the first and a positive result in the second sample.
negative controls containing only lysis buffer came out positive, even after hybridization. In all runs the standard with the lowest concentration of *M. leprae* DNA (i.e., 120 fg) showed an amplification product as judged by agarose gel analysis.

The total number of nasal swab specimens tested in PCR was 103. Twenty samples showed amplification products in PCR-A. The 83 negative samples were run in PCR-B jointly with 15 copies of the modified template. Of those samples, nine inhibited amplification of the modified template and the DNA was therefore purified. After purification, two showed amplification products in PCR-A, five were negative, but with amplification of the modified template, and two samples inhibited the amplification of the modified template in spite of purification. It could not be determined whether these two samples contained *M. leprae*.

**ELISA.** The average optical density of noncoated wells was 0.05 (n = 11), which made the cutoff value of the test 0.15. The lowest number of *M. leprae* that could be detected was 10^4. The optical density reached with this number of bacilli was 0.25. The four swab specimens from untreated MB patients that showed a positive BI on the nasal specimen smear showed the following optical densities: 0.47, 0.27, 0.19, and 0.01. Based on the cutoff value, the first three specimens could be interpreted as positive for the presence of phenolic glycolipid 1. The PCR results of the duplicate swab specimens were positive in the first three and negative in the last specimen.

**DISCUSSION**

Earlier studies employing the PCR have indicated that the method can be considered a useful tool for the rapid and sensitive detection of *M. leprae* in biological specimens (4, 7). To investigate the involvement of the nasal cavity in carriage of *M. leprae* among patients and healthy persons in areas of endemicity, we have further developed our previously described PCR (4, 9) for the detection of *M. leprae* in nasal swab specimens.

Initially, we found that components in the nasal mucosa could affect the amplification efficacy of *M. leprae* DNA (Fig. 1). Reduction of the sample size and addition of DMSO to the amplification reaction mixture resulted in amplification in all nasal swab specimens spiked with *M. leprae*. Previously, we have shown a similar effect of sample reduction on amplification efficiency by using biopsy specimens in PCR (4).

To check for inhibition that might occur in spite of the optimized conditions, we used an internal control. We chose a 531-bp modified fragment as the internal control because of its homology (it has only one base-pair mismatch) with the target DNA present in the clinical samples; it therefore required the same conditions for optimal amplification. Cloned in *Escherichia coli*, this modified template can be prepared in large quantities and used reproducibly over many experiments.

Apart from the false negativity, another major pitfall in PCR technology, that of false positivity due to cross-contamination of previously amplified DNA, has been avoided. Reamplification of carried-over amplicons has been prevented by the use of dUTP in combination with UDG (14). The effectiveness thereof was illustrated by the fact that none of the 72 negative controls run in 12 different PCR experiments was positive by gel analysis and hybridization.

In our study we found amplification products, which in our opinion is indicative of the presence of *M. leprae*, in 55% of

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**FIG. 3.** Agarose gel analysis of amplified *M. leprae* DNA in the amounts of 375 pg, 75 pg, 15 pg, 3 pg, and 600 fg (A, lanes 1 to 5); amplified modified template at 1.5 × 10^4 (A, lane 6), 1.5 × 10^3 (A, lane 12), 1.5 × 10^2 (C, lane 6), and 1.5 × 10 (C, lane 12) numbers of copies; amplified *M. leprae* DNA in the amounts of 375 pg, 75 pg, 15 pg, 3 pg, and 600 fg run jointly with the modified template at 1.5 × 10^4 (A, lanes 7 to 11), 1.5 × 10^3 (A, lanes 13 to 17), 1.5 × 10^2 (C, lanes 7 to 11), and 1.5 × 10 (C, lanes 13 to 17) numbers of copies. Restriction patterns after digestion with HaeIII are shown in the corresponding lanes of panels B and D. In lane 18 of both panels B and D, the undigested 531-bp fragment is shown.

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**FIG. 4.** Percentages of positive results in the PCR on nasal specimens from all groups. MB, untreated MB patients (n = 20); MB-T, MB patients under treatment (n = 5); OC, occupational contacts (n = 31); EC, endemic controls (n = 25); NC, nonendemic controls (n = 11).
untreated MB patients. Earlier studies have reported the presence of M. leprae in nasal discharges of leprosy patients (2, 3). In one study (3), an overall prevalence of 39% of M. leprae-containing discharges from 936 patients was reported. The true rates of nasal infection may be even higher than suggested by the PCR results. The pernasal swabs used for sample collection can contain only 20 μl of fluid. Furthermore, adequate sample collection is difficult for persons without special skill. It is likely that, if samples with a bigger volume than 20 μl had been collected and collection was carried out by a fully satisfactory method, the infection rates would have been higher.

On the other hand, it may not be the case that all patients carry bacilli in their noses. Furthermore, it should be considered that in this study the patients were clinically classified on the basis of the BI of their skin specimens. The size of the population of M. leprae in the nose does not necessarily reflect the bacterial density elsewhere in the body. Davey and Rees (3) reported that, in 16% of both early and late lepromatous patients, the BI was lower in the nasal discharge than in the skin.

In three of the four specimens with a positive nasal BI, M. leprae DNA could be detected by PCR. The negative result in PCR and ELISA suggests that the microscopic observation in one BI-positive specimen might have been due to staining of acid-fast bacilli other than M. leprae.

We did not find amplification products in the samples of treated patients. This is in agreement with earlier findings that chemotherapy lowers the bacillary content in the nose (3).

The percentage of PCR-positive nasal swab specimens found in occupational contacts (19%) was higher, although not significantly (Fisher exact test; P > 0.05), than the percentage (12%) found in the endemic control group. The percent positivity of the patient group was significantly higher (Yates corrected chi-square test; P < 0.05) than in both the group of occupational contacts and endemic controls. In none of the nonendemic controls were positive PCR results found. This might be an indication that carriage of M. leprae in the nose is indeed related to exposure to leprosy. Two persons with occupational contact had converted to positive results 1.5 years after the first (PCR-negative) sample collection. This is in agreement with earlier findings that prolonged exposure increases the risk of infection (8). The observation that two persons were positive by PCR twice after an interval of 1.5 years suggests that long-term carriage or/and reinfection is a possibility.

The results of this study provide evidence that a majority of MB patients are carrying M. leprae in their noses and that carriage of M. leprae occurs among healthy people living in an area where leprosy is endemic. Nasal carriage by healthy people might have an impact on leprosy control and thus be an important phenomenon from the public health point of view. New studies have to be undertaken to investigate whether and to what degree nasal carriage occurs in the general population of areas of endemicity and what might be the role of carriers in the maintenance of infection reservoirs and transmission of leprosy.

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