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Simplified PCR Detection Method for Nasal *Mycobacterium leprae*¹

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Following the introduction of the multidrug therapy (MDT) program in the 1980s, there has been a significant reduction in the estimated prevalence of leprosy worldwide, from around 12 million in the early 1980s, to approximately 1 million now. However, this decline in prevalence has not been mirrored by a concomitant fall in the observed incidence of the disease: over half a million new cases are still detected annually, a figure similar to that of 1985 (¹). This apparent discrepancy suggests that the widespread use of MDT is

having little impact on the transmission of leprosy. Although the causative agent of leprosy, *Mycobacterium leprae*, has long been known, the exact mode of transmission of the disease remains to be fully elucidated (¹). There appears to be few natural animal hosts of the bacterium in endemic areas: human-to-human contact is, therefore, thought to play a major role in providing a reservoir of infection. However, the long incubation period of the disease (often more than 5 years) and the low infection rate confound studies of transmission. Even in highly endemic areas, where the majority of the population is presumed to be exposed to *M. leprae*, less than 1% of the individuals usually develop clinical leprosy. In addition, the inability to culture the bacillus *in vitro* makes assessment of subclinical infection rates difficult.

It is thought that the nose is the usual site of primary infection with *M. leprae*, as a result of airborne infection (⁹). The advent of polymerase chain reaction (PCR) technology has afforded the opportunity to specifically detect small amounts of DNA, and a procedure, which can indicate the presence of DNA equivalent to as few as 20 *M. leprae* cells, has been developed by Hartskeerl, *et al.* (⁴). Studies using this technique have detected *M. leprae* DNA on swabs taken from the nasal mucosa of clin-

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ically normal individuals in a leprosy endemic population (^{5,11}). The significance of the presence of such DNA is as yet unresolved, but may represent a form of sub-clinical infection or transient carriage of *M. leprae*, which may in turn be important in the transmission of the disease. It may, therefore, be possible to use such PCR techniques to assess the load of transmission of leprosy within populations.

Performing basic research is often difficult in developing countries, where leprosy is endemic. Laboratory facilities are usually sparse and the supply of consumables, and reliable "cold chains" for the transport and storage of heat labile components of experiments, such as enzymes, etc., present problems in these areas. With these constraints in mind, we have developed a simplified but highly specific PCR method for the detection of *M. leprae* in an endemic population.

The method described here is simple, rapid, and robust. However for any such method, which is based on very sensitive PCR technique, it is essential to avoid false positive reactions. With this view we have studied samples obtained from a population from Norway, a leprosy non-endemic country. The purpose of the study was twofold: a) to evaluate specificity and accuracy of the method, and b) to study its suitability for testing of field samples. The results strongly suggest that this technique provides a potential means for large-scale screening of populations for the presence of sub-clinical nasal carriage of *M. leprae*.

MATERIALS AND METHODS

Subjects. As part of a large study aimed at addressing questions related to the transmission of leprosy, nasal swabs were collected from 219 individuals from Oslo, Norway. Swabs (Medical Wire and Equipment Company, Wiltshire, UK), dipped in sterile saline immediately prior to use, were passed along the base of the inferior turbinate until the posterior wall of the nasopharynx was encountered. Swabs were immediately chilled and transported to the laboratory for analysis. We also collected some swabs from untreated lepromatous patients at the Richardson Leprosy Hospital, Miraj, India. These latter swabs were used to optimize various parameters for the PCR reaction.

DNA extraction. The end of the swab was cut off, immersed in 100 µl of lysis buffer (1 mg/ml proteinase K in 100 mM Tris-HCl, pH 8.5, 0.05% Tween 20), and incubated under mineral oil (Sigma, UK) at 60°C overnight. Proteinase K was then inactivated by incubation at 97°C for 15 minutes.

PCR. The lysis solution was vortexed briefly to ensure mixing, and 2 µl taken for PCR analysis. PCR was performed using Ready-to-Go PCR beads (Pharmacia, UK), essentially according to the manufacturer's instructions, except that each primer was present at 50 ng per 25 µl reaction. *M. leprae*-specific primers, directed against part of the *M. leprae pra* gene, were: S13: 5'-CTC CAC CTG GAC CGG CGA T-3' and S62: 5'-Bio-GAC TAG CCT GCC AAG TCG-3'. These generated a 531 bp amplification product (²). Primer S62 was 5' biotinylated as indicated above for binding of PCR products to streptavidin-coated, 96-well plates. Positive control reactions contained 250 pg of purified *M. leprae* DNA, kindly provided by Dr. P. Brennan, Fort Collins, Colorado, U.S.A. In some experiments, primers to amplify a 212 bp region of the human β -globin gene were also included as an internal positive control to ensure that the PCR reaction had worked (PC04: 5'-CAA CTT CAT CCA CGT TCA CC-3' and GH20: 5'-GAA GAG CCA AGG ACA GGT AC-3', (¹²)). Reaction conditions were: initial denaturation 95°C, 5 min; 37 cycles of: 94°C, 2 min, 55°C, 1 min, 72°C, 2 min; and one cycle of 72°C for 10 min.

The samples were amplified with another set of *M. leprae*-specific primers (*rlep*) to cross check the results obtained with *pra* primers. The *rlep* primers (PS3: 5'-GGA CAC GAT TAG CGC GCA CGT-3' and PS4: 5'-Bio-TTG TGG TGG GCT GGT GGG GTG TGG-3') (⁶) were used at concentration of 50 ng each per reaction. Reaction conditions were: initial denaturation 95°C, 5 min; 37 cycles of: 94°C, 1 min, 65°C, 1 min, 72°C, 2 min; and one cycle of 72°C for 10 min. Primer PS4 was biotinylated at the 5' end. These primers generated a PCR amplification product of 455 bp.

PNA hybridization analysis. In an adaptation of the method described by Perry-O'Keefe, *et al.* (¹⁰), 10 µl from each 25 µl PCR reaction was mixed with 2 pmol of a

fluorescein-labeled PNA oligonucleotide probe (Perseptive Biosystems, UK) internal to the *M. leprae* *pra* PCR product (5'-Fluo-CCC AGC CAC GGT CCT-3') in a final volume of 40 µl, heat denatured at 95°C for 10 min, and allowed to cool slowly to room temperature over 2 hours. Ninety-six well, streptavidin-coated microtiter plates (Combiplate 8 Streptavidin, Labsystems, UK) were used for the ELISA. The DNA:PNA hybrid was added to these plates, incubated at room temperature (27°C ± 1°C) for 90 min, and washed at room temperature with Tris buffered saline with 0.1% Tween 20 (TBS/T, pH 7.4) 5–7 times. Plates were blocked with 200 µl of 2% BSA in TBS/T for 90 min at room temperature, and washed as previously. Fifty microliters of horseradish peroxidase (HRPO)-conjugated anti-fluorescein antibody (Sigma, UK, diluted 1:2000 in TBS/T) was added at room temperature for 90 min, the plates again washed as described, and 50 µl of substrate added (1 mg/ml O-phenylenediamine (OPD) in Phosphate-Citrate buffer with 0.1% H₂O₂; Sigma, UK). The reaction was terminated after 15 min incubation in the dark at room temperature, by the addition of 50 µl 3N HCl, and the optical density (OD) read at 490 nm on a Dynatech MR 5000 ELISA reader. The same procedure was followed for the samples amplified with *rlep* primers except that the fluorescein-labeled PNA probe used here was for the internal region of the *rlep* amplified product (5'-Fluo-CGC ACC TGA TGT TAT CCC-3').

Southern transfer. Ten microliters from each 25 µl PCR reaction was hybridized with PNA probe in 40 µl as described above. The whole sample was then loaded on 2% agarose gel, run at 75 V for approximately 2 hours, and immediately transferred onto positively-charged nylon membrane (Boehringer Mannheim, UK) by capillary transfer; standard Southern transfer denaturation and neutralization steps were not undertaken. The membrane was blocked overnight with constant agitation at room temperature in blocking solution (TBS/T with 2% BSA). It was then incubated in 25 ml anti-fluorescein antibody (1:2500 in blocking solution) for 2 hours at room temperature with constant agitation for detection, and washed 6–7 times with

TBS/T over 40 minutes to remove excess antibody. Color development was by incubation for 10–15 min at room temperature in 10 ml developing solution (0.38 mM BCIP [5-Bromo-4-chloro-3-indolyl phosphate] and 0.41 mM NBT [nitroblue tetrazolium] in alkaline phosphatase buffer [100 mM Tris, 100 mM NaCl, 5 mM MgCl₂; pH 9.5]). Development was terminated by the addition of EDTA in PBS at a final concentration of 0.2 mM.

Analysis of the PNA-ELISA results. To ensure the reproducibility and uniformity of the ELISA and positivity of the sample for *M. leprae*, stringent statistical criteria were applied for the acceptance of the results. The criteria for the acceptance of the results of ELISA were: a) the mean OD of the negative controls should be less than 0.3, b) the standard deviation in the OD values of the negative controls should be less than 0.2, c) the range (internal variation) in the OD values of the negative controls should be less than 0.25, and d) the difference between the OD values of the highest positive control and the mean negative control should be at least 1.000. These criteria were arrived at after analyzing the ELISA results for general background problems. The cut off point for defining PCR positivity using the ELISA method was determined by the formula: [Mean OD of negative control + (OD of highest positive control – Mean OD of negative control) × 0.3]. The results were cross checked by performing blots as described above.

RESULTS

We describe here a simple method for the rapid screening of PCR samples using PNA hybridization. This method was designed specifically to be simple and robust enough to be used in leprosy endemic areas.

PCR results. Nasal swabs were collected and processed as described, and PCR reactions performed on each of the samples. The use of 2 µl from 100 µl of lysate for the PCR reaction was determined to be optimal because we observed inhibition of the PCR reaction when larger volumes of lysate were used (data not shown). For optimization of PCR reaction, various parameters like annealing temperature, concentration of Mg²⁺, concentration of primers, requirement of dimethyl sulfoxide (DMSO) were

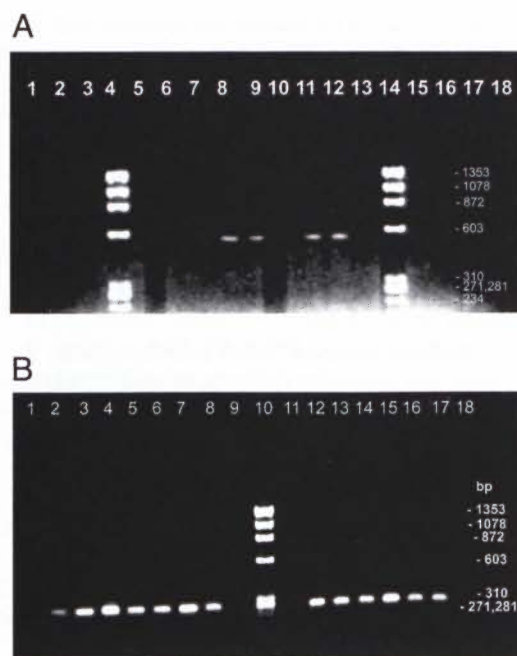


FIG. 1. **A** = Agarose gel electrophoresis of *M. leprae* PCR product (*pra* gene fragment) of size 531 bp. Lane 4—Markers (ΦX174 DNA/HaeIII fragments). Lane 6—Negative control (water control). Lane 8—Positive control (250 pG DNA). Lane 9—Positive control (250 pg DNA). Lane 11—Sample (nasal swab lysate from patient). Lane 12—Sample nasal swab lysate from patient). Lane 14—Markers (ΦX174 DNA/HaeIII fragments). **B** = Agarose gel electrophoresis showing human band (β-globin gene fragment) of size 210 bp. Lane 2–8—Nasal swab samples. Lane 10—Markers (ΦX174 DNA/HaeIII fragments). Lane 12–17—Nasal swab samples.

tested. The optimization of concentration for PNA for hybridization was also tested. The results are summarized in Table 1. For these experiments swabs collected from patients with lepromatous leprosy prior to treatment were used.

Figure 1A shows that a band of the appropriate size (531 bp fragment of *pra* gene) was produced by the PCR of lysate material from some samples of untreated lepromatous leprosy patients, when visualized on agarose gel. We postulated that human DNA would essentially always be present in the lysis solution, from cells sloughed off the nasal mucosa as the swabs were taken. By the inclusion of primers to amplify part of the human β-globin gene, we can, therefore, ensure that the PCR reaction had worked in the samples tested, on

TABLE 1. Optimization conditions for PCR reaction and PNA hybridization.

Parameter ^a	Optimum condition
Sample volume	2 µl
Mg ²⁺ concentration	1.5 mM
Primer concentration	50 ng
Annealing temperature	55°C
Dimethyl sulfoxide (DMSO)	Not required
PNA concentration required for hybridization	2 pmol

^a Various parameters for determining optimum PCR conditions were evaluated. Similarly PNA titration was carried out to determine its optimum concentration for hybridization.

the basis of a 212 bp human-specific band being observed. Amplification was observed in 94% (115 of 122) of the samples to which human β-globin primers were added (Figure 1B). However, these human-specific primers were not used routinely, because we observed that their addition resulted in diminution of the *M. leprae*-specific signal obtained in our detection system (data not shown). In practice, the use of the Ready-to-Go PCR beads minimizes inter-PCR run variability, and also reduces the likelihood of abortive reactions due to pipetting errors or failure of components. We are, therefore, confident that the few false negative samples resulted from the PCR reaction failing to work.

Although some samples were run on 1.5% agarose gels and appropriate bands observed (Fig. 1A), due to the design constraints of our study we did not routinely analyze samples by gel electrophoresis and subsequent Southern transfer, but instead the PNA system described was used. However, it was possible to visualize PCR products on gel. This is shown in Figure 2; bands of appropriate sizes can be seen in the gel of PCR samples which have not been prehybridized with PNA (Fig. 2, lane 5–8), but these bands cannot usually be visualized when samples are PNA hybridized (Fig. 2, lane 14–17). This is thought to be due to the inability of ethidium bromide stain to intercalate with the PNA : DNA hybrids, which are only double stranded for the short section to which the PNA probe is bound.

When prehybridized samples were transferred to nylon membrane and detected as described, bands of the appropriate size

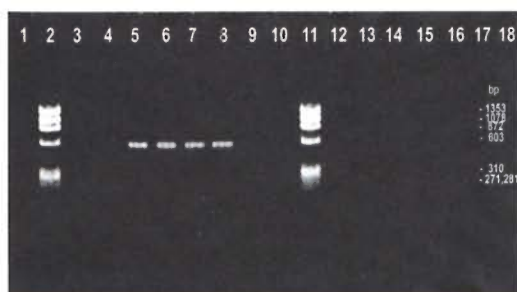


FIG. 2. Agarose gel electrophoresis of PNA-hybridized PCR product. Lane 2—Markers (Φ X174 DNA/*Hae*III fragments). Lane 4—Negative control (water control). Lane 5–6—Unhybridized PCR product (Positive control—250 pg DNA). Lane 7–8—Unhybridized PCR product (Sample from patient). Lane 11—Markers (Φ X174 DNA/*Hae*III fragments). Lane 13—Negative control (PNA-hybridized). Lane 14–15—PNA-hybridized PCR product (positive control). Lane 16–17—PNA-hybridized PCR product (sample).

(531 bp for *pra* amplicon and 455 bp for *rlep* amplicon) were observed (Fig. 3). These samples also showed positivity in the PNA plate by color development (data not shown). Note that gels of prehybridized PCR samples were not subjected to the standard Southern transfer denaturation and neutralization incubations, but instead were transferred immediately following electrophoresis. We found that such incubations abolished the PNA hybridization signal, probably due to cleavage of the linker molecule between the fluorescein moiety and the PNA by NaOH in the denaturation solution (personal communication, J. J. O'Leary, Cornell University, Ithaca, NY, U.S.A.), releasing the fluorescein label and rendering any bound PNA probe undetectable by our system.

The limits of detection by transfer and 96-well plate methods were observed to be similar by titration of *M. leprae*-positive control DNA, being approximately 125 fg, equivalent to 20 *M. leprae* bacteria (data not shown). However, as previously mentioned, for reasons of convenience and experimental design, samples in this study were routinely analyzed by the streptavidin-coated plate method, rather than by gel and transfer.

PCR survey results. We wished to assess the likelihood of false positive results arising in our technique. We, therefore, collected nasal swabs from 219 healthy indi-

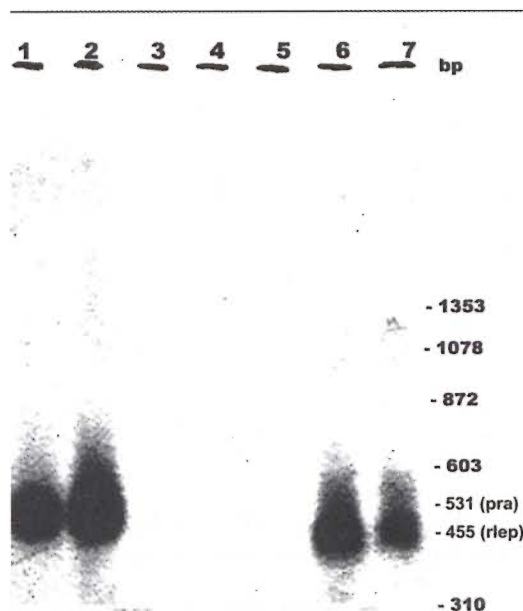


FIG. 3. Blot of the PCR product. Lane 1 & 2—*pra* band (531 bp fragment). Lane 6 & 7—*rlep* band (455 bp fragment).

viduals in Norway. Since Norway is a leprosy non-endemic country, it was expected that few if any of these samples would be positive for *M. leprae* DNA. When samples were tested in our laboratory in London, this proved to be the case: none of the samples had an OD higher than 0.289, so all were considered to be negative by our criteria. The mean OD of all the samples tested in India was 0.229.

These results suggested that the technique produces few false positive results, an acknowledged problem with PCR-based screening methods, due to the extreme sensitivity of the technique. To further assess the specificity of the technique, a small number of samples from Norway were randomly selected and had a known amount of *M. leprae* DNA added to them. All samples were then transported to our laboratory in India, where they were retested using the same technique under blind conditions. Of the 219 samples tested in this way, 6 were found to be PCR positive on this test when *rlep* primers were used for the DNA amplification. With *pra* primers, 5 swabs were positive. All the "positive" samples had been deliberately "spiked" with *M. leprae* DNA, and had previously given negative results in the PCR performed in London

TABLE 2. Results of PNA-ELISA on the spiked samples from Norway.

Sample no.	London OD ^a (before spiking)	Amount of <i>M. leprae</i> used for spiking samples (pg)	India <i>pra</i> OD ^b (after spiking)	India <i>rlep</i> OD ^b (after spiking)
33	0.155	125	2.241	2.5
124	0.107	125	1.89	2.5
129	0.125	62.5	0.225	2.5
151	0.123	250	2.366	2.5
190	0.077	62.5	1.515	2.5
230	0.173	250	1.824	2.5

^a Results of the testing (optical density shown by the samples when tested by PNA-ELISA) done at London. This testing was done with the original samples, i.e., before some samples were spiked with *M. leprae* DNA. Prior to PNA-ELISA, amplification was carried out by using *pra* primers.

^b Results of the testing (optical density shown by the samples when tested by PNA-ELISA) done in India. This testing was done blindly because some of the samples were spiked with *M. leprae* DNA. Prior to PNA-ELISA, amplification was carried out by using *pra* as well as *rlep* primers.

(see Table 2). None of the other samples from Norway was positive by this method.

DISCUSSION

It has previously been demonstrated that it is possible to detect the presence of small amounts of *M. leprae*-specific DNA on the nasal mucosa of apparently healthy individuals in an endemic population (⁷). We describe here the first use of PCR/PNA-ELISA system for the detection of *M. leprae* which can be used in an epidemiological field study. ELISA-based studies using DNA in detection of PCR products of other bacteria, such as that of McCarthy, *et al.*, have been done (⁸).

This method is designed for detection of *M. leprae* in endemic areas, and allows the simple and rapid screening of large numbers of samples. Due to the noninvasive procedure involved in the nasal swabs collection, subjects can easily be sampled quickly and safely in a temporary clinic, which can for example be set up in a room in a village school. Following the overnight lysis step, large numbers of swab samples can be processed immediately. The amplification reaction requires approximately 5.6 hours and 4.5 hours for *pra* and *rlep*, respectively. The PNA-ELISA requires approximately 6 hours. Thus, large numbers of PCR products can be rapidly tested by the PNA method described here.

Rapid screening of PCR samples was achieved using hybridization of a peptide nucleic acid (PNA) oligonucleotide, which is internal to the PCR product of interest. The PNA probe was labeled with fluores-

cein, facilitating its detection with an anti-fluorescein antibody conjugate in an ELISA-type reaction. An optical density (OD) value was, therefore, measured for each sample, with a high OD value indicating the presence of *pra* or *rlep*-specific amplicons bound to the plate. This allowed screening of large numbers of samples to be undertaken quickly and simply. Due to numerical output of the results, we are able to do statistical analysis of the results to determine cut off points to define PCR positivity. The data also can be used to evaluate the internal variation and the results of the negative controls can be used to decide the acceptability of the results and to take care of any background problems associated with the ELISAs. The intensity of the color developed in the ELISA plate was related to the amount of template DNA. Hence, this method can be suitably modified to make it semi-quantitative. Earlier van der Vliet, *et al.*, have reported a calorimetric microtiter method for the detection of amplified *M. leprae* DNA (¹²). They have reported that the optical densities showed a logarithmic-linear relationship with the amount of template DNA.

In the design of this method, we have been careful to take into account the problems associated with working in a leprosy endemic area. The use of commercially prepared PCR Ready-to-Go beads in this procedure may seem expensive, but it obviates the need for a reliable "cold chain" to keep labile reaction components (such as *Taq* polymerase) refrigerated in order to maintain their integrity during transport, and also

reduces the staff time needed to prepare the PCR reactions. By using Ready-to-Go PCR beads, we were also able to eliminate much of the inter-PCR variability, which can make results in population screening experiments difficult to interpret. Beads were re-suspended in a premixed solution containing the PCR primers at the required concentration. This largely eliminated potential pipetting errors with regard to primers. We found these beads to be robust despite sub-optimal transport conditions (temperature fluctuations, storage, customs delays), and experienced only one instance of PCR failure due to a ruptured package.

In our study, we adapted the procedure of Perry-O'Keefe, *et al.*, for the PNA detection of PCR products (¹⁰). A peptide nucleic acid (PNA) oligonucleotide probe, which is internal to the PCR product of interest was hybridized to each of the PCR samples. These PNA:DNA hybrids were bound to streptavidin-coated, 96-well plates by the biotinylated 3' PCR primer, and the presence of the bound PNA probe determined by an ELISA-type detection of a 5' fluorescein moiety attached to the probe. There are a number of advantages to using PNA in this system. PNA binds to denatured DNA with greater avidity than does the complementary DNA strand, but it is more sensitive to base mismatches than DNA:DNA hybrids; the specificity of detection is, therefore, increased using a PNA probe in comparison to an equivalent DNA probe (³), thereby reducing the likelihood of false positive results. Probe binding and washing conditions are also simplified by the use of PNA; due to its high binding affinity, low salt buffers can be used. Another advantage of PNA probes lies in their length: PNA probes are shorter than their DNA equivalents (on average 15 bases as opposed to 20 bases) and although their sequence is, therefore, less unique, it is observed that probes of shorter length tend to be more sequence specific in their binding (¹⁰).

The specificity of the detection procedure which we describe here is further enhanced by the use of the PNA ELISA in the analysis of the samples: any amplicons derived from non-specific priming in the PCR reaction which bind to the plate will not be expected to contain the internal sequence specific to the PNA probe, and would,

therefore, not register as positive in our assay. High throughput of samples for analysis is also facilitated by the use of this 96-well plate system; all of the samples in this study could be rapidly and simply analyzed on a small number of plates. Additional advantages of the procedure include a reduction in the subjectivity of the screening procedure as compared to one based on the presence of a band on an agarose gel; variations in detection due to loading and transfer of gels are also eliminated. Since a numerical optical density (OD) value is assigned to each readout sample, these simplify the statistical analyses of the results obtained.

The results described here indicate that the technique is not subject to producing significant numbers of erroneous results. The addition of human β -globin gene primers indicates that false negatives due to the failure of the PCR reaction are rare. We are, however, aware that little is known about the distribution of *M. leprae* on the nasal mucosa, and hence it is possible that in some cases false negatives may arise from the swabs not sampling an area of the membrane which has bacteria on the surface.

In order to examine the question of false positives, an acknowledged problem with PCR based techniques, we obtained and tested swabs from residents of Norway. Because Norway is a leprosy non-endemic country, these swabs were expected to be largely negative for *M. leprae*: this was the case when they were tested in our laboratory in London. However, on addition of *M. leprae* DNA to a small number of the samples, and retesting all of them in our lab in India under blind conditions, we found that all the 6 spiked swabs out of total of 219 samples tested were PCR positive with the *rlep* primers, whereas with the *pra* primers we failed to detect one spiked sample. Both systems showed all other samples to be negative, confirming our assumption that these swabs would be negative since they were from subjects from a leprosy non-endemic country. If any of the unspiked swabs had been positive, this would have meant that either nasal *M. leprae* were present in a non-endemic subject, an unlikely scenario, or a false positive result. In that case we would have had to reconfirm the positivity with another sample from the

same subject as well as to carry out external quality control to rule out false positivity.

A major factor, which must be borne in mind when designing such tests for use in leprosy endemic areas, is the cost of the test. The largest single element of cost in the process is in the Ready-to-Go PCR beads: as enumerated earlier, such a system introduces a number of significant advantages as compared to "standard" PCR procedures, such as obviating the need for a reliable "cold chain," and largely eliminating potential inter-run pipetting errors. While at present this technology is too costly to be considered for widespread use as a part of leprosy control programs, we believe that it can give useful information suitable for the design of control methods for the interruption of transmission.

The techniques described here are simple, rapid and robust, and will have effectiveness in the rigorous environment in which leprosy fieldwork is carried out. We are currently engaged in an extensive project to study nasal carriage in a much larger sample population in a leprosy endemic area, which will also include the sampling of dust from a variety of sites within houses with PCR-positive subjects and control houses without PCR-positive subjects, for analysis by PCR to try to detect environmental contamination of house dust with *M. leprae*. We already have carried out external quality control checks for some samples in three different labs from three different countries.

SUMMARY

We report here a simplified method for the detection of nasal carriage of *Mycobacterium leprae*. DNA extracted from nasal swabs was analyzed by PCR, and *M. leprae* specific amplicons detected by means of a novel peptide-nucleic-acid-ELISA (PNA-ELISA) method. Parameters for the method were established using swabs taken from untreated lepromatous leprosy patients.

We have developed this method to study nasal carriage in endemic populations. However, due to the sensitivity of PCR based techniques, we wished to assess the possibility of false positive samples arising in our method. We therefore examined samples taken from individuals in Norway, a country non-endemic for leprosy, using our technique. A total of 219 nasal swabs were collected and tested in our laboratory in

London. All of these were found to be negative by our criteria. In order to corroborate our results, and also to assess the specificity of the method, a small number of these samples were randomly selected, and a known amount of *M. leprae* DNA added to them. All 219 samples were then retested using the same techniques under "double blind" conditions in our laboratory in India. All of the samples to which *M. leprae* DNA had been added were successfully identified by this method whereas all other swabs were negative. Taken together, these results suggest that the technique described here is simple, sensitive, and specific for use in large-scale epidemiological studies. This study, part of the larger MILEP 2 study, represents the first use of a PNA-PCR method for an epidemiological study of infection. The method using PNA-ELISA is significantly simpler and more rapid than gel based detection methods. The supply of laboratory consumables and overall detection procedure were simplified and standardized by use of PCR Ready-to-Go beads.

RESUMEN

Se describe un método para la detección de *Mycobacterium leprae* en el exudado nasal de portadores de la lepra. El DNA extraído de los hisopos impregnados con moco nasal se analizó por la reacción en cadena de la DNA polimerasa (PCR), y los amplicones específicos para *M. leprae* se detectaron usando un novedoso método descrito como péptido-ácido-nucleico-ELISA (PNA-ELISA). Los parámetros para el método se establecieron utilizando los exudados tomados de pacientes con lepra lepromatosa sin tratamiento.

El método se desarrolló para el estudio de portadores nasales en poblaciones endémicas. Debido a la alta sensibilidad de las técnicas basadas en la PCR, decidimos establecer la frecuencia de resultados falsopositivos obtenidos con este método. Para esto examinamos muestras tomadas de individuos en Noruega, un país no endémico para la lepra. Se colectaron 219 muestras de exudados nasales y se examinaron en nuestro laboratorio en Londres. Todas estas muestras resultaron negativas, según los criterios preestablecidos. Para corroborar estos resultados, y también para establecer la especificidad del método, tomamos, al azar, un pequeño número de estas muestras y las contaminamos con cantidades conocidas de DNA de *M. leprae*. Después, todas las muestras (219) se volvieron a probar usando la misma técnica en un estudio "doble ciego" en nuestro laboratorio en la India. Todas las muestras a las que se había adicionado DNA de *M. leprae* fueron exitosamente identificadas por este método mientras que el resto de las muestras permanecieron negativas. En conjunto, estos resultados indican que la técnica de-

escrita aquí es simple, sensible, y específica para su uso en gran escala en estudios epidemiológicos. Este estudio, el cual es parte de un estudio MILEP 2 más extenso, describe el uso, por primera vez, de un método de PNA-PCR para el estudio epidemiológico de una entidad infecciosa. El método usando PNA-ELISA es significativamente más simple y más rápido que los métodos de PCR en gel. El suministro de reactivos y consumibles, y el procedimiento global de detección, fueron simplificados y estandarizados por el uso de esferas para PCR "listas para usarse."

RÉSUMÉ

Une méthode simplifiée de détection dans le nez des porteurs sains de *Mycobacterium leprae* est présentée. L'ADN fut extrait d'écouvillons de fosses nasales, amplifié par réaction de polymérase en chaîne (PCR) et les amplicons spécifiques de *M. leprae* furent détectés au moyen d'une nouvelle méthode de peptide-acide nucléique-ELISA (PNA-ELISA). Les paramètres méthodologiques furent établis en utilisant des écouvillonnages de fosses nasales de patients lépreux lépromateux encore non traités.

Nous avons développé cette méthode afin d'étudier l'importance du réservoir nasal dans les populations endémiques. Cependant, du fait de la haute sensibilité des techniques basées sur le PCR, nous avons souhaité évaluer l'existence d'échantillons faussement positifs. Nous avons donc testé des échantillons prélevés à partir d'individus de Norvège, un pays indemne de la lèpre, avec notre technique. Un total de 219 écouvillonnages fut recueilli et testé dans notre laboratoire à Londres. Ils furent tous déclarés négatifs par nos critères. Afin de corroborer nos résultats, ainsi que pour évaluer la spécificité de notre méthode, un nombre limité de ces échantillons, sélectionnés au hasard, reçut une quantité connue d'ADN isolé de *M. leprae*. La totalité des 219 échantillons fut testés à nouveau en "double aveugle" et en utilisant les mêmes techniques dans notre laboratoire indien. Tous les échantillons, qui reçurent de l'ADN de *M. leprae*, furent positivement identifiés par cette méthode tandis que les autres écouvillons furent négatifs. Globalement, ces résultats supportent la notion que la technique décrite ici est simple, sensible et spécifique pour une application à des études épidémiologiques de grande envergure. Cette étude, qui fait partie intégrante de la grande étude MILEP 2, représente la première application de la méthode PNA-PCR à une étude épidémiologique d'infection. La méthode utilisant la PNA-ELISA est significativement plus simple et plus rapide que celles utilisant les méthodes de détection sur gel d'agarose. L'utilisation de consommables de laboratoires et l'ensemble du protocole de détection furent simplifiés et standardisés par l'utilisation de microbilles pour PCR prêtes à l'emploi.

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REFERENCES

1. CREE, I. A. and SMITH, W. C. S. Leprosy transmission and mucosal immunity: towards eradication? *Lepr. Rev.* **69** (1998) 112-121.
2. DE WIT, M. Y. L., DOUGLAS, J. T., MCFADDEN, J. and KLATSER, P. R. Polymerase chain reaction for detection of *Mycobacterium leprae* in nasal swab specimens. *J. Clin. Microbiol.* **31** (1993) 502-506.
3. EGHOLM, M., BUCHARDT, O., CHRISTENSEN, L., BEHRENS, C., FREIER, S. M., DRIVER, D. A., BERG, R. H., KIM, S. K., NORDEN, B. and NIELSEN, P. E. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365** (1993) 566-568.
4. HARTSKEERL, R. A., DE WIT, M. Y. L. and KLATSER, P. R. Polymerase chain reaction for detection of *Mycobacterium leprae*. *J. Gen. Microbiol.* **135** (1989) 2357-2368.
5. HATTA, M., VAN BEERS, S. M., MADJID, B., DJUMADI, A., DE WIT, M. Y. and KLATSER, P. R. Distribution and persistence of *Mycobacterium leprae* nasal carriage among a population in which leprosy is endemic in Indonesia. *Trans. R. Soc. Trop. Med. Hyg.* **89** (1995) 381-385.
6. JAMIL, S., WILSON, S. M., HACKET, M., HUSSAIN, R. and STOKER, N. G. Colorimetric PCR method for the detection of *M. leprae* in skin biopsies from leprosy patients. *Int. J. Lepr.* **62** (1994) 512-520.
7. KLATSER, P. R., VAN BEERS, S., MADJID, B., DAY, R. and DE WIT, M. Y. L. Detection of *M. leprae* nasal carriage in leprosy endemic population. *J. Clin. Microbiol.* **31** (1993) 2947-2951.
8. MCCARTHY, J. S., ZHONG, M., GOPUNAT, R., OTTESEN, E. A., WILLIAMS, S. A. and NUTMAN, T. B. Evaluation of a polymerase chain reaction-based assay for diagnosis of *Wuchereria bancrofti* infection. *J. Infect. Dis.* **173** (1996) 1510-1514.
9. PALLAN, M. J. and MCDERMOTT, R. D. How might *Mycobacterium leprae* enter the body? *Leprosy Review* **57** (1986) 289.
10. PERRY-O'KEEFE, H., YAO, X. W., COULL, J. L., FUCHS, M. and EGHOLM, M. Peptide nucleic acid pre-gel hybridization: an alternative to Southern hybridization. *Proc. Natl. Acad. Sci. USA* **93** (1996) 14670-14675.
11. RAMAPRASAD, P., FERNANDO, A., MADHALE, S., RAO, J. R., EDWARD, V. K., SAMSON, P. D., KLATSER, P. R., DE WIT, M. Y. L., SMITH, W. C. S. and CREE, I. A. Transmission and protection in leprosy: indications of the role of mucosal immunity. *Lepr. Rev.* **68** (1997) 301-315.
12. VAN DER VLIET, G. M. E., HERMANS, C. J. and KLATSER, P. R. Simple colorimetric microtiter plate hybridization assay for detection of amplified *Mycobacterium leprae* DNA. *J. Clin. Microbiol.* **31** (1993) 665-670.
13. WORLD HEALTH ORGANIZATION. WHO status report (1997). Geneva: World Health Organization, 1997. WHO/LEP/97.4.