A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens

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Abstract

An RNA-based assay is an additional molecular tool for leprosy diagnosis and determination of the viability of leprosy bacilli. To simplify RNA detection, a one-step reverse transcriptase PCR (RT-PCR) was established and evaluated. RNA and DNA could be isolated simultaneously. With the use of *Mycobacterium leprae*-specific primers targeting a 171-bp fragment of the *M. leprae* 16S RNA gene, RT-PCR resulted in detection of *M. leprae* in both slit skin smears and skin biopsy specimens. To enhance the positive signal, a digoxigenin-labeled DNA was developed, and successfully detected the amplified RT-PCR product. The method is sensitive, as it could detect one leprosy bacillus. When it was used directly on skin specimens collected from leprosy patients, 34 of 36 multibacillary (MB) and 13 of 24 paucibacillary (PB) cases showed positive results. The assay was also effective in monitoring bacterial clearance in leprosy patients during chemotherapy; after treatment with the multidrug therapy for 6 months, resulting in bacterial clearance, 16 of 36 MB patients and three of 24 PB patients tested were still positive for the 16S rRNA gene of *M. leprae*, suggesting the advisability of a more prolonged treatment course. This form of RT-PCR is of value in terms of simplicity and sensitivity in identifying *M. leprae* in routine skin specimens, especially when acid-fast bacilli are not discernable.

Introduction

Leprosy, a result of infection by *Mycobacterium leprae*, is a chronic disease of skin and nerve, and is of special significance because it can progress to peripheral neuropathy and permanent progressive deformity, leading to the social consequences of discrimination and stigma (Brycesson & Pfaltzgraff, 1990). Diagnosis based on clinical symptoms compounded by social contexts, such as poor public relations, difficulty in accessing health delivery systems, and neglect of self-care, may lead to late detection, resulting in disease progression with multiple and/or severe disabilities. For patients whose clinical signs cannot be readily defined, histopathologic features and demonstration of acid-fast bacilli (AFB) in skin and nerve biopsy specimens are considered as definite evidence of the disease. The universal implementation of diagnosis based largely on physical characteristics is probably partly responsible for the continuing high new case detection rate in the midst of declining prevalence (Brycesson & Pfaltzgraff, 1990; International Leprosy Association, 2002).

Simple, reproducible, specific and sensitive methods for the detection of *M. leprae* or evidence of infection are required. The insensitive method of identification of AFB in skin smears is the only laboratory-based diagnostic method used, and is primarily used to confirm diagnosis and to define the various clinical aspects of the disease (Brycesson & Pfaltzgraff, 1990). There are a few useful serologic tests for the diagnosis of leprosy (Fine *et al.*, 1988; Hastings *et al.*, 1988), primarily based on *M. leprae*-specific phenolic glycolipid I (Cho *et al.*, 1983); however, these show limitations in the detection of paucibacillary (PB) forms of leprosy, being mostly applicable to the multibacillary (MB) forms of the disease.

PCR amplification in various forms has been developed and used for leprosy diagnosis, and has proven to be highly

sensitive and specific, being able to detect small numbers of *M. leprae* organisms in slit skin smears, skin biopsy specimens, nasal swab specimens and environmental samples (Harstskeerl *et al.*, 1989; Woods & Cole, 1989; Hackel *et al.*, 1990; Plikaytis *et al.*, 1990; Williams *et al.*, 1990; Cox *et al.*, 1991; de Wit *et al.*, 1991; Arnoldi *et al.*, 1992; Pattyn *et al.*, 1992; Yoon *et al.*, 1993; Jamil *et al.*, 1994; Wichitwechkarn *et al.*, 1995; Scollard *et al.*, 1998). Recently, reverse transcriptase PCR (RT-PCR) has been recognized as a rapid and sensitive method for the detection of RNA of *M. leprae* (Kurabachew *et al.*, 1998). Whereas nucleic acid amplification tests targeting rRNA genes give poor predictions of bacterial viability, rapidly degradable mRNA may have potential in this respect (Gabrielle *et al.*, 1994). However, detection of mRNA is relatively difficult, because mRNA is labile and few copies are present in each cell compared to those of rRNA genes. In this study, we focused on developing a simplified, sensitive RT-PCR method, based on *M. leprae* rRNA genes, for routine use in confirmation of leprosy diagnosis, monitoring of leprosy chemotherapy, and identification of relapsing patients.

Materials and methods

Skin tissue samples

The Institutional Ethics Committee of the Ministry of Public Health, Nonthaburi, Thailand approved the study for human research. After informed consent was obtained, slit skin smears and skin biopsy specimens, as part of the routine collection of skin samples for staining of AFB and histopathologic investigations, were collected from leprosy patients visiting two skin clinics in Bangkok, a leprosy hospital in Samutprakarn Province, the Leprosy Regional Center 5 in Nakornrachaseema, the Leprosy Regional Center 6 in Khon Kean, and the Northeastern Hospital of Infectious Diseases, Khon Kean, Thailand. Newly diagnosed, untreated leprosy patients were classified as PB or MB, according to clinical features and bacterial index (BI); the BI was determined on the basis of the number of detectable AFB in slit skin smears, and grading was performed according to Ridley's logarithmic scale (Ridley, 1958, 1964). With these standard criteria, PB patients were defined as those having a negative BI but fewer than five distinctive clinical appearances diagnostic for leprosy; the MB patients were those presenting with five or more leprosy skin lesions regardless of BI and any patients positive for BI in slit skin smear examination. Patients were further classified into indeterminate (I), tuberculoid (TT), borderline tuberculoid (BT), borderline lepromatous (BL), and lepromatous (LL) leprosy according to the Ridley–Joplin scale (Ridley & Jopling, 1966). For the BT type, patients were defined as BT(–) or BT(+), depending on the presence or absence of AFB. Sixty of these untreated patients were enrolled in the study. Slit skin smears and skin biopsy specimens were taken according to standard procedures (World Health Organization, 1987) before and at 6 months after the start of the WHO multidrug therapy (MDT) regimen. At the time points of skin slit smear collection, BI was determined and reported as average BI values obtained from six sites of slit skin smears for MB patients and three sites for PB patients. Based on clinical signs and enumeration of AFB in slit skin smears, 24 of these patients were classified as having PB leprosy [2 I, 11 TT and 11 BT(–)] and 36 were classified as having MB leprosy [2 BT(–), 9 BT(+), 21 BL and 4 LL]. Upon collection, portions of slit skin smears or 6-mm punch biopsy specimens were frozen immediately at $-20\,^{\circ}C$ prior to shipping on ice to Sasakawa Research Building, Bangkok, for RNA extraction. As controls, parts of skin tissue from five normal skin volunteers and 10 patients with skin diseases other than leprosy, such as sarcoidosis and leishmaniasis, were collected.

Extraction of RNA and DNA

Prior to manipulation of clinical samples, comparative RNA extraction procedures were applied to *M. smegmatis* and subsequently to *M. leprae* isolated from infected mouse footpad and leprosy skin biopsy specimens, using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH), TRIzol (Gibco & Invitrogen Corporation, Gaitherberg, MD) or the RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturers' protocols, with modifications. Skin biopsy punch specimens were cut into small pieces with sterile scissors and manually ground in a glass tissue homogenizer in the presence of 200 μL of RNase-free water containing 100 U of RNase inhibitor (Amersham Biosciences, Alameda, CA). In order to prepare homogenates from slit skin smears, three to six blades per subject, used for slit skin smear excisions, were placed in a 1.5-mL tube, and 100 μL of RNase-free water (treated with diethyl pyrocarbonate) was added to resuspend skin tissue. The tube, containing the blades, was then sonicated in cold water (*c.* 4 °C) for 10 min using an ultrasonicator bath (VWR, Aurora, CA) at a frequency of 40 kHz, and lysozyme ($10\,mg\,mL^{-1}$) and proteinase K ($3\,mg\,mL^{-1}$) (Amersham Biosciences) were then added. After incubation at 37 °C for 10 min, 100 μL of cell homogenate was transferred to a 1.5-mL tube, and 900 μL of the TRI reagent was added. Chloroform (200 μL) was subsequently added to the homogenate mixture, which was then shaken vigorously and centrifuged at 12 000 ***g*** at 4 °C for 15 min to produce aqueous, interphase and organic layers. The RNA-containing aqueous phase was carefully removed, leaving the interphase and organic layers for further DNA extraction. RNA was precipitated by the addition of 2.5 volumes of absolute cold ethanol, 0.1 volume

of 3 M sodium acetate, and 1 μL of 20 mg mL^{-1} glycogen (Boehringer Mannheim, Mannheim, Germany). After centrifugation at 14 000 ***g*** at 4 °C for 15 min, the RNA pellet was recovered, washed in 75% ethanol, dried at room temperature, and solubilized in 30 μL of RNase-free water. The purified RNA was frozen at −20 °C until it was used for RT-PCR analysis. RNAs isolated with the TRI reagent from *M. leprae* derived from experimentally infected mouse footpads were used as controls.

Mycobacterium leprae DNA was isolated from the organic and interphase layers obtained from the initial homogenate. Absolute cold ethanol (300 μL) was added to the combined organic layer/interphase layer to precipitate DNA, which was sedimented by centrifugation at 2000 ***g*** at 4 °C for 10 min. The resulting DNA pellet was washed twice with 1 mL of 0.1 M sodium citrate in 10% ethanol and once in 75% ethanol. DNA, recovered after further centrifugation, was allowed to air dry, solubilized in 50 μL of 8 mM NaOH, and stored at −20 °C. Purified *M. leprae* DNA from armadillo-derived *M. leprae* (provided through NIH, NIAID Contract AI-25469 to Colorado State University) was included as a standard control.

RT-PCR

The first set of primers, P1 and P3, are genus-specific, which allowed the amplification of 231-bp fragments of 16S rRNA genes from various species of mycobacteria (Arnoldi *et al*., 1992). A second set of primers, P2 and P3, specifically amplify an internal *M. leprae*-specific 171-bp fragment of the above PCR product (Cox *et al*., 1991). The sequences of the primers were as follows: CTC AGT GTA GCC CAG GAT GC, *M. leprae* 16S rRNA gene P1 primer (positions 9–18 of the DNA sequence); AGA GTT TGA TCC TGG CTC AG, *M. leprae* 16S rRNA gene P2 primer (positions 69–91 of the DNA sequence); and CAT CCT GCA CCG CAA AAA GCT T, *M. leprae* P3 primer (positions 218–239 of the DNA sequence). All oligonucleotides used as primers were synthesized in the BioService Unit, BIOTEC Center, National Service and Technology Development Agency, Thailand.

Reverse transcription and PCR were carried out sequentially in a single tube using Ready-To-Go RT-PCR beads (Amersham Biosciences); each room-stable bead contained M-MuLV reverse transcriptase, RNase inhibitor, buffer, nucleotides and *Taq* DNA polymerase. The only additional reagents needed to complete the reaction mixtures were 20 pmol of each primer, and 30 μL of RNA template in RNase-free water. The RT-PCR reaction, in a total volume of 50 μL, was conducted in a programmable thermal machine (Astec, Fukuoka, Japan), using the same cyclic profile for the two sets of 16S rRNA gene primers. The thermal program involved reverse transcription at 45 °C for 30 min, followed by initial denaturation at 94 °C for 5 min. The amplification comprised 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The final extension was set at 72 °C for 10 min. Negative controls, which contained all reaction components except RNA template, were included in all tests to detect carryover contamination.

To determine whether transcribed RNA or contaminating DNA was amplified by this protocol, purified nucleic acids were subjected to DNase (Amersham Biosciences) and RNase (Qiagen) treatment prior to cDNA synthesis and PCR, as previously described (Huang *et al*., 1996). To ensure that amplicons derived from RT-PCR originated from RNA, a control reaction containing RNase was performed. Briefly, extracted RNA was treated with 1 μL of 10 mg mL^{-1} RNase in the absence of RNase inhibitor. After incubation at 37 °C, the reaction was terminated by heat inactivation at 80 °C for 10 min. The resulting RNase-treated RNA was used as control template in parallel with untreated RNA, which was then added directly to the RT-PCR reaction mixture.

The amplicons obtained from RT-PCR were analyzed for the 171-bp fragment of the 16S rRNA gene of *M. leprae* by 2.0% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, pH 8.0, 44 mM boric acid, 1 mM EDTA) (Sambrook *et al*., 1989). Electrophoresis was conducted at 100 V for 1 h. Gels were stained with ethidium bromide and visualized by UV transillumination.

Southern blot hybridization

The DNA-labeled sequence targeting the 231-bp fragment of the *M. leprae* 16S rRNA gene was labeled with digoxigenin by random prime labeling, as described by the manufacturer (Boehringer Mannheim). Prior to using the probe in Southern blot hybridization for detection of the specific PCR amplicon, it was tested for efficiency by assessing hybridization of the probe to genomic DNA of *M. leprae* and *M. smegmatis*. For Southern blot hybridization experiments, the PCR products were electrophoresed through 2% agarose gel. After denaturation in 0.5 M NaOH/1 M NaCl for 30 min, DNA was transferred overnight by capillary diffusion to nylon membrane (Boehringer Mannheim) and fixed by UV crosslinking. The blotted membranes were then prehybridized for 1 h, at 55 °C, in buffer containing 5 × SSC (prepared from the stock solution of 20 × SSC, which contained 3 M NaCl plus 0.3 M sodium citrate, pH 7.0), 1% blocking solution (Boehringer Mannheim), 0.1% *N*-laurosylsarcosamine sodium salt, and 0.02% sodium dodecyl sulfate. Subsequently, the membranes were hybridized to heat-denaturated digoxigenin-labeled DNA probe at 55 °C overnight, and washed in 5 × SSC (1 × SSC is 0.15 M NaCl containing 0.015 M sodium citrate with 0.1 M sodium dodecyl sulfate) twice for 10 min at room temperature. Sequential washing in 1 × SSC with 0.1 M sodium dodecyl

sulfate at 55 °C for 15 min was performed. *Mycobacterium leprae* DNA labeled with the probe was finally detected colorimetrically using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and the color-forming substrates BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium), according to the manufacturer's instructions (Boehringer Mannheim).

Specificity and sensitivity of the test

The specificity of the test for *M. leprae* was evaluated using *M. leprae*-specific primers, P2 and P3. Mycobacteria used to analyze the specificity of the test included *M. leprae*, *M. avium*, *M. bovis*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. smegmatis* and *M. tuberculosis*. Another 10 bacterial species – *Streptococcus agalactiae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Bacillus cereus*, *Corynebacterium ulcerans*, *Salmonella typhimuri*, *Shigella dysentery*, *Vibrio parahaemolyticus* and *Escherichia coli* – that represented the most commonly isolated species recovered in the clinical microbiology laboratory were added to further test specificity. All mycobacteria were cultured on 7H11 agar, except *M. leprae*. The other bacteria were grown on blood agar under aerobic conditions. Cell numbers equivalent to 10^8 colony forming units (CFU) of these organisms were processed for RNA extraction. The resulting RNA from various organisms was subjected to RT-PCR and electrophoresis. The specificity of the test for leprosy was also analyzed using skin tissues collected from 10 patients who had skin diseases other than leprosy, and skin specimens as part of skin biopsy specimens from five patients who had undergone reconstitutive surgery, but had normal skin. RNA was extracted from all skin specimens, and subjected to RT-PCR, as described above.

The sensitivity of the test was determined using *M smegmatis* and *M. leprae*. Suspensions of *M. leprae* were prepared from the 6-mm skin biopsy specimens collected from leprosy patients. The number of AFB in the homogenate was determined by acid-fast staining (Shepard & McRae, 1968), leading to a stock suspension containing *c.* 1×10^6 bacilli mL^{-1}. Serial dilutions of *M. leprae* were prepared in RNase-free water, and then subjected to RNA extraction and RT-PCR analysis as described above. *Mycobacterium smegmatis* was also used as a surrogate strain to determine the lowest number of bacilli that the assay was capable of detecting. The analysis of sensitivity was conducted by preparing serial 10-fold dilutions of the bacterial suspensions in RNase-free water containing RNase inhibitor, lysozyme and proteinase K. An aliquot of 900 μL of the TRI reagent was added to 100-μL volumes of the bacterial suspensions containing, *c.*, the following numbers of organisms: 10^5, 10^4,10^3, 10^2, 10 and one. Samples were processed for RNA extraction as described above, and aliquots of the cell suspension were plated for colony counting.

Direct DNA PCR

The PCR assay targeting the DNA sequence of the *M. leprae* 16S rRNA gene was developed with the two sets of primers described above. The PCR reaction was performed in a total volume of 50 μL containing 2 U of *Taq* polymerase (Molecular Research Center), 2 mM dNTP mix (Gibco & Invitrogen Corporation), 20 pmol of each set of primers, 10% dimethyl sulfoxide, PCR buffer, 30 μL of either extracted DNA, standard *M. leprae* DNA, or sterile distilled water for clinical specimens, positive controls, and negative controls, respectively. The thermal cycle was programmed as described above, and the PCR product was detected by agarose gel electrophoresis or Southern blot hybridization.

Results

Extraction of *M. leprae* RNA and DNA

A simple procedure for RNA isolation from *M. leprae*-infected skin tissue capable of providing high yields of RNA for subsequent RT-PCR amplification was desired. Three different methods of RNA extraction were conducted using *M. smegmatis* as a surrogate, and also *M. leprae*; all allowed successful amplification (Fig. 1a and b). The extraction procedures were also applicable to small quantities of cells; as few as 10 *M. smegmatis* organisms could be detected (Fig. 1a). The use of the TRI reagent to isolate RNA involved minimal sample manipulation, and the yield was comparable to that obtained with the RNeasy kit, which also offers equal ease in isolation of RNA. In addition, the TRI reagent enabled simultaneous isolation of DNA and RNA from the same skin specimen. As a result, the extraction of RNA and DNA with the TRI reagent was selected and applied to the analysis of the entire range of clinical specimens.

Practical specificity of primers P2 and P3

Of the two sets of oligonucleotide primers applied to amplify two regions of the *M. leprae* 16S rRNA gene (Cox *et al.*, 1991; Arnoldi *et al.*, 1992; Kurabachew *et al.*, 1998), P1 and P3 are genus-specific and resulted in the amplification of a 231-bp fragment spanning positions 9–239 of the 16S rRNA gene of *M. smegmatis* (Fig. 1a). A second pair of primers, P2 and P3, target the same gene, but are *M. leprae*-specific, leading to amplification of the internal 171-bp fragment spanning position 69–239 of the 16S rRNA gene (Cox *et al.*, 1991; Arnoldi *et al.*, 1992) (Fig. 2a–c). The P1 and P3 primers enabled us to work with the surrogate strain, *M. smegmatis*, which can be cultivated and is readily quantitated, and hence the 231-bp fragment was amplified

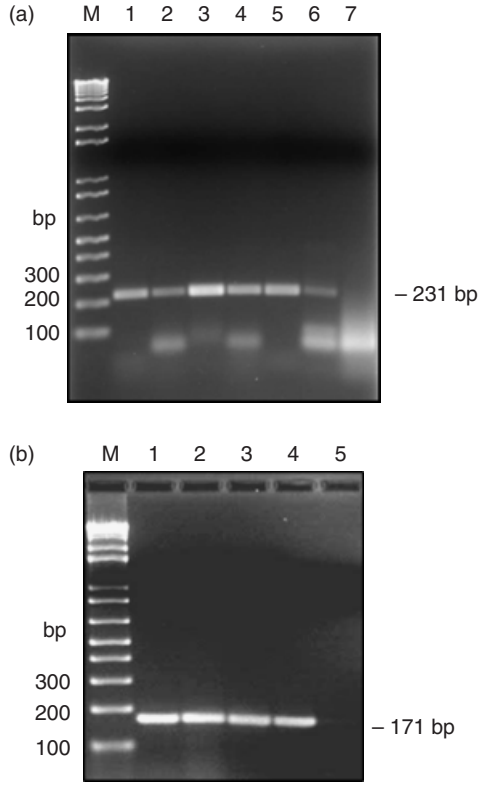

Fig. 1. RT-PCR after three different RNA extraction procedures. (a) Extraction of RNA from *Mycobacterium smegmatis*. RNA was extracted from the surrogate mycobacterium, *Mycobacterium smegmatis*, with the RNeasy kit, the TRI reagent and TRIzol solution. The resulting RNA was subjected to DNase treatment, cDNA synthesis and PCR. Lanes: M, DNA markers; 1, RNeasy kit, 10^5 CFU; 2, RNeasy kit, 10^2 CFU; 3, TRI reagent, 10^5 CFU; 4, TRI reagent, 10^2 CFU; 5, TRIzol, 10^5 CFU; 6, TRIzol, 10^2 CFU; 7, negative control (no RNA templates). (b) Extraction of RNA from *Mycobacterium leprae*. RNA was extracted from *Mycobacterium leprae*. Lanes: M, DNA marker; 1, positive control (RNA extracted from mouse footpad infected tissue using TRI reagent); 2, RNeasy kit; 3, TRI reagent; 4, TRIzol; 5, negative control.

with the P1–P3 primer pair (Fig. 1a). The 'ready-to-use' commercially available beads for conducting reverse transcription and PCR in one tube enhanced reproducibility and simplicity of manipulation.

To evaluate species specificity, RNA from *M. smegmatis*, *M. tuberculosis* and other mycobacteria was purified, and subjected to RT-PCR and electrophoresis. No PCR product was observed with either agarose gel electrophoresis or Southern blot analysis when nucleic acids from as much as 10^8 CFU of 10 non-*M. leprae* mycobacteria (Fig. 2a) and 10 other selected bacteria were applied to the P2 and P3 primers (data not shown); thus, the P2 and P3 primers amplified a 171-bp product only in the presence of the RNA template from *M. leprae* (Fig. 2a and b). There was no detectable band of PCR product when the RNA template derived from patients with other skin diseases and healthy volunteers was assayed (Fig. 2b and c). It can be concluded that the specificity of the test relies on the P2–P3 primer set.

Practical sensitivity of RT-PCR

The sensitivity of the assay was first evaluated by applying the P1 and P3 primers to *M. smegmatis*. Cell numbers as low as 10 CFU were detectable by RT-PCR when RNA was extracted with the RNeasy kit, the TRI reagent or TRIzol (Fig. 1a). When the cell number was less than 10 CFU, extraction of RNA with the TRI reagent, followed by RT-PCR, allowed detection of the RT-PCR product (Fig. 3a). The sensitivity of the assay for the detection of *M. leprae* was also assessed. Leprosy bacilli were isolated from skin biopsies collected from MB leprosy patients, RNA was extracted from serial dilutions of known number of bacillary cells, and cDNA was synthesized from RNA; this was followed by PCR amplification and electrophoresis. An RT-PCR product was detected even when only one bacillus was present (Fig. 3b). To ensure that the RT-PCR product was not due to contamination of RNA with chromosomal DNA, the product was treated with DNase; this resulted in no reduction in the RT-PCR signal (Fig. 4). Likewise, treating the RT-PCR product with RNase resulted in its removal (Fig. 4).

In an attempt to increase the efficiency of detection of the RT-PCR product, a DNA probe spanning positions 9–239 of the 16S rRNA gene of *M. leprae* was tested for its sensitivity, using *M. smegmatis* as a surrogate strain. The hybridization signal was easily recognized down to the level of 10 AFB (Fig. 5a). Agarose gel electrophoresis or Southern blot hybridization allowed detection of *M. leprae* RNA in slit skin smears (Fig. 5b) and skin biopsy specimens from leprosy patients (Fig. 5c).

Slit skin smear and skin biopsy samples are difficult to obtain, and there is a limit to specimen number and size. Accordingly, it is beneficial to isolate DNA and RNA from the same specimens. The TRI reagent, successful in the isolation of RNA, was applicable to the extraction and recovery of *M. leprae* DNA, through precipitation from the organic phase and interphase with ethanol. The alkaline conditions needed for DNA extraction did not seem to interfere with the subsequent PCR reaction (Fig. 5d).

RT-PCR and clinical leprosy

The applicability of the assay to the detection of *M. leprae* in the clinical context was also evaluated. Of 60 patients seeking medical attention at the time of visiting, 24 were classified as PB and 36 as MB (Table 1). Of these 36, 24 were AFB-positive, and therefore a BI could be assigned. The results of RT-PCR from skin tissues collected from the TT and BT leprosy patients showed that 13 of 24 were positive (Table 1). Histopathologic examinations were conducted on eight of these 24; five were positive, and three of these five

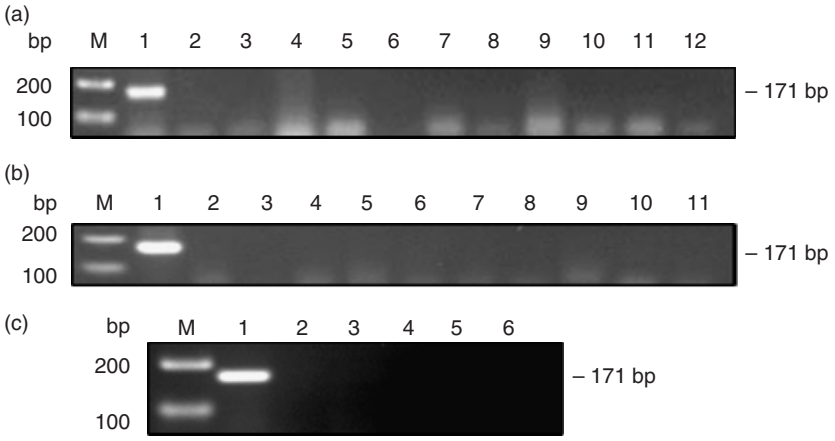

Fig. 2. Specificity of the test. (a) Specificity for detection of *Mycobacterium leprae*. RNA was extracted from *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and other mycobacteria. The resulting RNA was subjected to DNase treatment, cDNA synthesis, and PCR, using the P2 and P3 primer pair, specific for a 171-bp sequence of the *Mycobacterium leprae* 16S rRNA gene. Lanes: M, DNA markers; 1, *Mycobacterium leprae*-positive; 2, *Mycobacterium leprae*-negative; 3, *Mycobacterium avium*; 4, *Mycobacterium bovis*; 5, *Mycobacterium fortuitum*; 6, *Mycobacterium gordonae*; 7, *Mycobacterium intracellulare*; 8, *Mycobacterium kansasii*; 9, *Mycobacterium marinum*; 10, *Mycobacterium scrofulaceum*; 11, *Mycobacterium smegmatis*; 12, *Mycobacterium tuberculosis*. (b) Specificity for detection of leprosy. RNA was extracted from skin specimens of leprosy patients and from patients with other skin diseases. The resulting RNAs were subjected to RT-PCR using the P2 and P3 primers. The 171-bp fragment specific for *Mycobacterium leprae* was detected by electrophoresis. Lanes: M, DNA marker; 1, leprosy; 3–11, other skin diseases. (c) RT-PCR analysis of healthy skin specimens. Lanes: M, DNA marker; 1, positive control; 2–6, normal skin specimens.

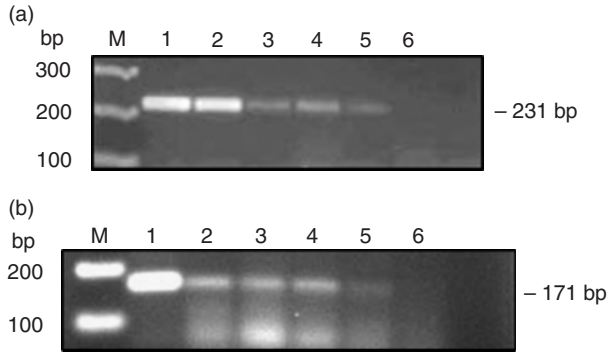

Fig. 3. Sensitivity of RT-PCR. (a) Determination of the sensitivity of RT-PCR using *Mycobacterium smegmatis* as a surrogate for *Mycobacterium leprae*. Serial 10-fold dilutions of a known number of cells were made in 1 mL of RNase-free water. Enumeration of cell numbers in bacterial dilutions was performed by plating on 7H11 agar. Cells were subjected to RNA extraction, RT-PCR, and electrophoresis. Lanes: M, DNA markers; 1, positive control; 2, 10^3 CFU; 3, 10^2 CFU; 4, 10 CFU; 5, less than 10 CFU; 6, negative control. (b) Determination of the sensitivity of RT-PCR for detection of *Mycobacterium leprae*. In parallel, the sensitivity of RT-PCR for detection of *Mycobacterium leprae* was determined using *Mycobacterium leprae* clinical isolate. A series of 10-fold dilutions of leprosy bacilli was made in 0.1% diethyl pyrocarbonate in water. RNA was extracted and subjected to cDNA synthesis, PCR and electrophoresis. Lanes: M, DNA marker; 1, *Mycobacterium leprae*-positive control; 2, 10^3 bacteria; 3, 10^2 bacteria; 4, 10 bacteria; 5, one bacterium; 6, negative control.

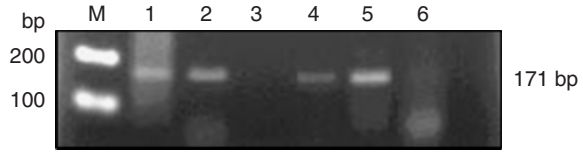

Fig. 4. The effects of RNase and DNase treatments on the isolated nucleic acids. RNA samples were subjected to treatment with nucleases (DNase or RNase) prior to cDNA synthesis and PCR. The RT-PCR was performed using the specific P2 and P3 primers. Lanes: M, DNA marker; 1, no DNase or RNase treatment; 2, DNase treatment; 3, RNase treatment; 4 and 5, from multibacillary (MB) patient 1 and MB patient 2 treated only with DNase; 6, negative control. The conditions for DNase and RNase digestion were first optimized to ensure that optimal digestions took place in the given environment.

were positive by RT-PCR. However, of the three that were negative by histopathology, one was positive by RT-PCR, and two of the three were negative by both methods. On the basis of this evidence, the diagnostic positivity rate for PB leprosy, both by histopathology and by RT-PCR, was about 63%.

Mycobacterium leprae was detected by RT-PCR in 34 of the 36 MB patients, and 16 of the 34 MB patients remained positive for the 16S rRNA gene of *M. leprae* for 6 months after the start of leprosy treatment with MDT. It is important to note that two RT-PCR-negative MB patients, of the 36, were BI-negative. In PB patients, the RT-PCR analysis gave positive results in three of 24 patients at 6 months after the start of leprosy therapy with MDT (Table 1). Apparently, the 16S rRNA gene signal declined during and after chemotherapy in both MB and PB patients.

Discussion

Currently, the routine diagnosis of leprosy is still largely based on clinical manifestations, with the results of AFB

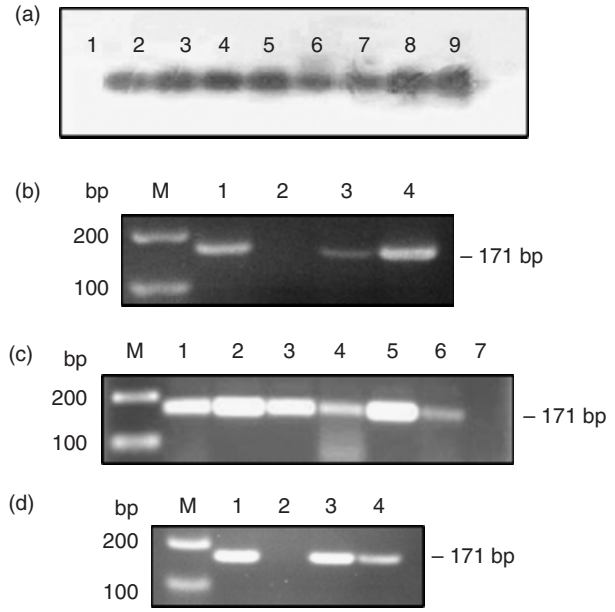

Fig. 5. Detection of DNA and RNA from skin specimens of leprosy patients by RT-PCR. (a) Southern blotting and hybridization. A digoxigenin-labeled DNA probe targeting the 231-bp fragment of the 16S rRNA gene of *Mycobacterium leprae* and *Mycobacterium smegmatis* was developed and used to detect the RT-PCR products. Lanes: 1, negative control; 2–9, RT-PCR aliquot of *Mycobacterium smegmatis* bacterial dilutions containing 10–10^8 bacilli. (b) Detection of the *Mycobacterium leprae* 16S rRNA gene in slit skin smears from leprosy patients. Slit skin smears from each patient were subjected to RNA extraction, RT-PCR and electrophoresis. Lanes: M, DNA markers; 1, positive control (known clinical sample containing *Mycobacterium leprae*); 2, negative control (skin tissue extract); 3 and 4, aliquots of the RT-PCR product amplified from slit skin smears from two patients with bacterial indices of +2 and +4, respectively. (c) Detection of the 16S rRNA gene of *Mycobacterium leprae* in skin biopsy specimens. Skin biopsy specimens were collected from leprosy patients and subjected to RNA extraction, RT-PCR and electrophoresis. Lanes: M, DNA marker; 1, RNA-positive control with DNase treatment; 2, RNA-positive control without DNase treatment; 3–6, aliquots of the RT-PCR product amplified from skin biopsy specimens from four patients; 7, negative control without RNA template. (d) DNA-PCR for detection of *Mycobacterium leprae* in skin biopsies. For confirmation of diagnosis, DNA-PCR was performed using DNA extracted from the same source of skin specimens. The resulting DNA was subjected to direct DNA PCR and electrophoresis. Lanes: M, DNA markers; 1, positive control containing *Mycobacterium leprae* DNA; 2, negative control without *Mycobacterium leprae* DNA; 3 and 4, DNA PCR from two leprosy patients.

staining of slit skin smears only sometimes being applied as confirmation or for classification of leprosy type. At least two of the following characteristics of leprosy skin lesions are required for diagnosis: loss of sensation; thickening of peripheral nerve fibers; and the presence of AFB in smears of skin lesions (Talhari, 1996). In many cases, a definitive diagnosis cannot be made, leading to difficulty in making treatment decisions, and thus making long-term patient follow-up necessary. A more definitive test is therefore desirable (Nalin *et al.*, 1999). Molecular methods are the preferred means, and involve a variety of techniques, such as amplification of *M. leprae*-specific repetitive sequences (Yoon *et al.*, 1993), nested primer amplification (Estrada *et al.*, 1988), and RT-PCR (Kurabachew *et al.*, 1998). Although PCR for DNA is widely accepted for the detection of infection, an important limitation is its inability to distinguish between viable and nonviable organisms. On the other hand, the methodology of RT-PCR has the potential to detect not only *M. leprae*, but also its viability status.

Table 1. RT-PCR analysis for detection of *Mycobacterium leprae* RNA in clinical samples from multibacillary (MB) and paucibacillary (PB) leprosy patients before and after chemotherapy

	No. positive cases/No. tested (%)			
			RT-PCR	
Leprosy	Histopathology	Bacterial index	Before treatment	After 6 months of treatment
MB	No data	24/36	34/36 (94)	16/36 (44)
PB	5/8	0/24	13/24 (54)	3/24 (12.5)

Discrimination between the viability and nonviability of *M. leprae* is important for correct prognoses of leprosy patients on treatment, determination of drug resistance, or identification of relapse. Therefore, the ability of tests to distinguish between viable and nonviable organisms is required. It has been noted that an RNA-based test is likely to reflect only nucleic acids from living organisms, as the turnover rate of RNA is high, particularly in prokaryotes, with an estimated half-life of 3 min (Albert *et al.*, 1989). Because mRNA is degraded rapidly upon cell death, detection of mRNA, if not total RNA, should allow an estimation of cell viability (Kurabachew *et al.*, 1998). In addition, because *M. leprae* apparently contains only one copy of the 16S rRNA gene (Sela *et al.*, 1989), but *c.* 4000 molecules of 16S rRNA in each cell (Estrada *et al.*, 1988), PCR detection of rRNA should provide a sensitive assay of the presence of *M. leprae* itself (Kurabachew *et al.*, 1998). Although the question of total RNA, notably mRNA, as a marker of cell viability was not addressed in the present work, the potential of 16S rRNA gene detection as a solution to some of the shortcomings of leprosy diagnosis was addressed.

There have been several publications on the use of different kinds of PCR to detect *M. leprae* in clinical specimens. Jadhav *et al.* applied TRIzol for RNA extraction, following this by two steps of RT-PCR to detect *M. leprae* in slit smears from leprosy patients (Jadhav *et al.*, 2005). The extraction procedures involving TRIzol and the TRI reagent are similar. The difference in manipulation of skin specimens may influence the detection results. Kurabachew *et al.*

developed an RT-PCR targeting a 171-bp segment of the 16S rRNA gene of *M. leprae*, and demonstrated that it could be applied to clinical specimens (Kurabachew *et al.*, 1998). In this latter work, the RNA was extracted with a gunidinium isothiocyanate solution, phenol–chloroform extraction and isopropanol precipitation. RT-PCR was performed in two sequential steps involving cDNA synthesis and PCR. One of the challenges in this present work was to develop rapid, efficient and simple methods for recovery of nucleic acids (DNA or RNA) from test specimens and amplification using RNA of *M. leprae* as template in the presence of host materials. We describe here an extraction procedure requiring *c.* 2 h to obtain separated *M. leprae* RNA as well as DNA from clinical specimens. The TRI reagent combines phenol and guanidine thiocyanate in a monophasic solution; therefore, it facilitates the immediate and effective inhibition of RNase activity, and hence RNA in tissue specimens is not degraded when cells are resuspended and stored in TRI reagent. Nevertheless, specimens were processed in a timely manner, because freezing may be detrimental to cell viability (de Wit *et al.*, 1991). It appears from our study that 1 week storage at $-70\,^{\circ}C$ is acceptable, and allows some flexibility in the timing of specimen processing.

With use of the TRI reagent, an improvement over earlier methods of total RNA extraction, *M. leprae* RNA could be extracted from both infected mouse footpads and skin specimens (Chomezynski & Sacchi, 1987; Chomezynski, 1993). However, some modifications had to be incorporated into the TRI reagent procedure on account of high yields of *M. leprae* RNA obtained from skin specimens. Accordingly, cells were vigorously lysed by sonication, and this was followed by digestion with lysozyme and proteinase K. Subsequently, skin samples were subjected to homogenization or lysis in the TRI reagent. Upon addition of the TRI reagent to the cell homogenate, RNA remained exclusively in the aqueous phase, whereas DNA was in the interphase fraction and, with proteins, in the organic fraction. Phase separation was achieved by the addition of chloroform. DNA was sequentially precipitated from the interphase and organic layers with ethanol. Mild alkaline treatment replaced water, as it ensures full solubilization of DNA. The resulting DNA upon amplification could be the basis of confirmation of leprosy diagnosis. These methods for RNA and DNA extraction using commercially available reagents as described here are sufficiently simple and robust to be used in routine leprosy diagnosis.

In general, the reactions of reverse transcription and PCR are performed in two steps. Initially, a first-strand cDNA is synthesized. The resulting cDNA is then transferred to another tube containing *Taq* DNA polymerase and PCR buffer, where the reaction is subjected to multiple cycles of denaturation, annealing and elongation, resulting in the exponential amplification of the target cDNA. The RT-PCR methodology reported here is a simple one, as reverse transcription and PCR were carried out sequentially in the same tube. Ready-To-Go RT-PCR beads simplify this process to a single tube and single reaction procedure. The bead format of RT-PCR significantly reduced the amount of pipetting, thereby increasing the reproducibility of the techniques and minimizing the risk of contamination and RNA degradation. All components required for both reactions are added during the setup step, and there is no need to add additional components once the reaction has started. Primers P1–P3 employed in this method have been well validated and evaluated in previous work (Cox *et al.*, 1991; Arnoldi *et al.*, 1992; Kurabachew *et al.*, 1998). It was reported that primer P2 annealed to an AT-rich sequence within the 12-bp insertion that is found in the variable region of the 16S rRNA gene. This insertion is unique to the DNA nucleotide sequence of the *M. leprae* 16S rRNA gene (Cox *et al.*, 1991; Teske *et al.*, 1991). Our work confirms that the RT-PCR targeting the 171-bp fragment spanning positions 69–239 of the *M. leprae* 16S rRNA gene is specific in operational terms.

Detection of *M. leprae* RNA has been proposed as a promising tool for rapid detection and measurement of the viability of *M. leprae* (Kurabachew *et al.*, 1998; Chae *et al.*, 2002). A positive signal reflected the presence of viable organisms, whereas negative results could be explained by the absence of viable bacilli in collected specimens or the presence of reaction inhibitors. Alternatively, given our inability to achieve 100% sensitivity, it is possible that no bacilli were present in the aliquot of processed tissue, as the numbers of bacilli were very low in the skin specimens collected from this group of patients. This observation exposes a limitation of the test.

As the leprosy bacilli cannot be cultivated and mouse footpad inoculation is difficult, the detection of RNA might be useful for assessment of leprosy therapy. The study of Chae *et al.* demonstrated that RT-PCR and DNA PCR for the 18-kDa protein of *M. leprae* were effective in assessing the efficacy of MDT, and 63.6% of leprosy patients showed positive PCR results after MDT treatment for 12 months (Chae *et al.*, 2002). We have shown that the RT-PCR for the *M. leprae* 16S rRNA gene can be applied to monitor the efficacy of leprosy treatment. A reduction in RNA was detected with this simplified RT-PCR, which showed a reduction or disappearance of the 171-bp amplicon after 6 months of MDT for leprosy. In this study, 18 of 34 (52.4%) MB and 10 of 13 (76.9%) PB patients with RT-PCR-positive results became RT-PCR-negative after 6 months of MDT. A correlation between the BI and RT-PCR results at 6 months of MDT could be seen in 72% of BI-positive, MDT-treated patients. Prolonged monitoring in this group of MB patients may lead to definite conclusions on the correlation between BI and detection of RNA. The remaining *M. leprae*, as

detected by RT-PCR, indicated the need for a longer course of treatment.

In conclusion, this RT-PCR is a sensitive, specific and simple method for the rapid detection of *M. leprae* in skin specimens. Because of the simplicity, short processing time, and economy, the method holds promise as an additional tool for leprosy diagnosis and follow-up during leprosy treatment, and for distinguishing relapsing patients from those with leprosy reaction.

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