RESEARCH ARTICLE

LightCycler™ real-time PCR for rapid detection and quantitation of *Mycobacterium leprae* in skin specimens

Janisara Rudeeaneksin[1], Sopa Srisungngam[1], Pathom Sawanpanyalert[1], Thaverit Sittiwakin[2], Sirirat Likanonsakul[3], Supannee Pasadorn[4], Prasit Palittapongarnpim[5], Patrick J. Brennan[6] & Benjawan Phetsuksiri[1]

[1]Mycobacteria Laboratory, Sasakawa Research Building, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; [2]Raj-Pracha-Samasai Institute, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand; [3]Bamrasnaradura Infectious Disease Institute, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand; [4]Sirindhorn Hospital, Ministry of Public Health, Nonthaburi, Thailand; [5]Department of Microbiology, Mahidol University, Bangkok, Thailand; and [6]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

Correspondence: Benjawan Phetsuksiri, Mycobacteria Laboratory, Sasakawa Research Building, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, 11000. Tel.: +662 580 1567; fax: +662 965 9700; e-mail: benjapsk@health.moph.go.th

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Abstract

Diagnosis of leprosy is usually based on clinical features and skin smear results including the number of skin lesions. *Mycobacterium leprae* is not cultivable and bacterial enumeration by microscopic examination is required for leprosy classification, choice in choosing and monitoring chemotherapy regimens, and diagnosis of relapse. However, detection and quantification using standard microscopy yields results of limited specificity and sensitivity. We describe an extremely sensitive and specific assay for the detection and quantification of *M. leprae* in skin biopsy specimens. Primers that amplified a specific 171-bp fragment of *M. leprae* 16S rRNA gene were chosen and specificity was verified by amplicon melting temperature. The method is sensitive enough to detect as low as 20 fg of *M. leprae* DNA, equivalent to four bacilli. The assay showed 100% concordance with clinical diagnosis in cases of multibacillary patients, and 50% of paucibacillary leprosy. The entire procedure of DNA extraction and PCR could be performed in *c.* 3 h. According to normalized quantitative real-time PCR, the patients in this study had bacilli numbers in the range of 1.07×10^2–1.65×10^8 per 6-mm^3 skin biopsy specimen. This simple real-time PCR assay is a facile tool with possible applications for rapid detection and simultaneous quantification of leprosy bacilli in clinical samples.

Introduction

The global burden of leprosy, a disease caused by *Mycobacterium leprae* infection, is declining but the disease has remained a public health problem in several countries (WHO, 2007). Access to disease information, diagnosis and treatment with multidrug therapy (MDT) remain key elements in the strategy to control leprosy (WHO, 1998, 2007). *Mycobacterium leprae* has never been cultured in the laboratory. The identification of acid-fast bacilli (AFB) in skin smears is the only readily available laboratory-based diagnostic method, but it is insensitive. PCR has provided sensitive and specific methods for the detection of a number of pathogenic organisms including *M. leprae* (Woods & Cole, 1989; Pikaytis *et al.*, 1990; Williams *et al.*, 1990; Jamil *et al.*, 1993; Wichitwechkarn *et al.*, 1995; Van der Vliet *et al.*, 1996; Kampirapap *et al.*, 1998; Scollard *et al.*, 1998; Kang *et al.*, 2003; Phetsuksiri *et al.*, 2006). Recently, real-time PCR was introduced to enable rapid detection and quantification of many pathogens in a specific and sensitive way, but few such tests were applied to *M. leprae* (Kramme *et al.*, 2004; Martinez *et al.*, 2006). The platform LightCycler™ real-time PCR (LC-PCR) (Roche Diagnostics, Indianapolis, IN) method is attractive as it combines rapid thermal cycling in specially designed glass capillaries and the specificity of amplicons can be verified immediately postamplification using melting curve analysis (Witter *et al.*, 1997; Rasmussen *et al.*, 1998). Detection is based on measuring fluorescence signals at the end of the extension phase of each PCR cycle following binding of the SYBR Green to PCR products. The

advantages of LC-PCR technology also include speed and the ability to monitor continuously the progress of the PCR reaction in each sample per cycle (Witter *et al*., 1997; O'Mahony & Hill, 2002, 2004). In this study, a simple LC-PCR assay based on SYBR Green was developed for the rapid detection and simultaneous quantification of *M. leprae*, and shown to provide the most accurate, convenient and sensitive means to date for evaluation of leprosy in skin biopsy specimens.

Materials and methods

Specimen collection and processing

Punch skin biopsies (6 mm^3) were collected following informed consent from newly diagnosed leprosy patients seen at two skin clinics, in Bangkok and Sirindhorn Hospital in Khon Kaen Province, Thailand. The patients were diagnosed and classified as having paucibacillary (PB) or multibacillary (MB) leprosy by clinicians based on clinical features and bacterial indices (Ridley & Jopling, 1966). The number of skin lesions, according to WHO criteria for leprosy classification, was also taken into account. The bacterial index (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, 1964). At the time point of slit skin smear collection, BI was determined and reported as average values obtained from six sites for multibacillary patients and three sites for paucibacillary patients. With these criteria, paucibacillary patients were defined as those having a negative BI but fewer than five distinct clinical appearances indicative of leprosy; the multibacillary patients were defined as those presenting with five or more skin lesions regardless of BI and any patients positive for BI in slit skin smear examination. As shown in Table 1, the total number of patients recruited into this study was 66, defined as 14 lepromatous leprosy (LL), 20 borderline lepromatous (BL), 25 borderline tuberculoid (2 BT+; 23 BT−), six tuberculoid (TT) and one indeterminate (I). For the BT type, patients were defined as BT(−) or BT(+), depending on the presence or absence of AFB. Skin biopsy specimens were obtained before leprosy treatment, and stored at −20 °C before shipment on ice to Sasakawa Research Building for DNA extraction, amplification and microscopic enumeration of AFB.

The processing of skin biopsy specimens has been described (Phetsuksiri *et al*., 2006).

DNA was extracted using a commercial DNA extraction kit (Flexigene®, Qiagen; Valencia, CA) with modifications, as follows: 100 μL of homogenized skin tissue was incubated at 65 °C for 15 min in 100 μL of lysis buffer (included in the kit) containing a final concentration of 300 μg mL^{-1} of proteinase K (Qiagen). After inactivating the enzyme at 95 °C for 10 min, DNA was precipitated with isopropanol, washed in 70% ethanol, dried and resuspended in 50 μL of TE buffer consisting of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA and maintained at −20 °C for PCR analysis.

Table 1. The efficiency of LC-PCR for the detection of *Mycobacterium leprae* in skin specimens of leprosy patients*

Leprosy type	No. of positive samples detected (%)	Total samples
Multibacillary	38 (100)	38
Subtype LL	14	14
Subtype BL	20	20
Subtype BT+	2	2
Subtype BT−	2	2
Paucibacillary	14 (50)	28
Subtype BT−	11	21
Subtype TT	2	6
Subtype I	1	1

*LL, lepromatous leprosy; BL, borderline leprosy; BB, borderline borderline; BT, borderline tuberculoid, a five group system according to Ridley and Jopling's scale in leprosy classification. Paucibacillary, Patients showing negative smears at all sites or fewer than five leprosy skin lesions. Multibacillary, Patients showing positive smears at any site or presenting with five or more skin lesions regardless of BI. BT patients defined as BT(−) or BT(+), depending on the presence or absence of AFB. TT, tuberculoid; I, indeterminate.

PCR

The target of amplification was a specific region of the *M. leprae* 16S rRNA gene based on the two primers (Cox *et al*., 1991; Kurabachew *et al*., 1998; Phetsuksiri *et al*., 2006). The oligonucleotide sequences were AGA GTT TGA TCC TGG CTC AG (the P2 primer, position 69–91 of the 16S rRNA gene) and CAT CCT GCA CCG CAA AAA GCT T (P3 primer, position 218–239 of the16S rRNA gene).

For conventional PCR, the mixture consisted of reaction buffer, 200 μM of dNTPs, 1 U *Taq* DNA polymerase, 10 μL of DNA template and 10 pmol of specific primers in a total volume of 25 μL. Amplification conditions have been described (Cox *et al*., 1991). The amplified DNA was detected using 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, pH 8.0, 44 mM boric acid and 1 mM EDTA) (Sambrook *et al*., 1989), followed by ethidium bromide staining.

For LC-PCR, a single tube fluorogenic real-time PCR assay was conducted using a LightCycler™ machine. LC-PCR conditions were optimized based on conventional PCR parameters. The optimized conditions involved a commercial ready-to-use reaction mixture of the FastStart SYBR Green DNA Master mix (Roche Diagnostics) that contained FastStart *Taq* DNA polymerase, SYBR Green dye, dNTP mixture, reaction buffer and 25 mM $MgCl_2$ at a final concentration of 4 mM, 10 pmol of pair primers and 2 μL

of DNA template all in a final volume of 20 μL. The reaction mixture was subjected to real-time PCR cycling by hot start followed by multiple cycles of denaturing at 95 °C for 5 s, annealing at 60 °C for 5 s and extension at 72 °C for 8 s. Fluorescence acquisition was set up to occur within the F1 channel during the annealing phase of each cycle; this was followed by a melting program of 75–95 °C to characterize amplified products. The analysis of fluorescence was carried out using LC software version 3.01 (Roche Diagnostics).

For determination of LC-PCR sensitivity, serial 10-fold dilutions of known concentrations of purified *M. leprae* DNA and known number of leprosy bacilli were prepared from skin suspensions and subjected to LC-PCR. To prepare dilutions of known number of bacteria, a volume of homogenates was 10-fold serially diluted with sterile distilled water. Aliquots of 10 μL of cell dilutions were spread over 8-mm diameter circles on clean microscopic slides, dried, fixed and stained using the Zeihl–Neelsen method and enumerated for the numbers of AFB by microscopic examination (magnification, × 1000 in 64 oil-immersion fields) (Shepard & McRae, 1968). The original concentration of bacillary cells was calculated, leading to a stock suspension containing 2×10^6 bacilli mL^{-1}. Each dilution of known cell number was subjected to DNA extraction and LC-PCR as described above.

The specific primers used were examined extensively as described (Cox *et al.*, 1991; Kurabachew *et al.*, 1998; Phetsuksiri *et al.*, 2006). As the melting curve profile replaced agarose gel analysis to identify amplicons in this assay, a melting temperature of a single LC-PCR product obtained was determined using the mode of melting curve analysis. To confirm the specificity of primers, DNA from related mycobacteria including *Mycobacterium smegmatis*, and other bacteria were tested using LC-PCR. As controls, genomic DNA isolated from skin tissue of 10 patients with skin diseases other than leprosy (nonleprosy), such as sarcoidosis and leismaniasis, were also assessed for false-positive results. The efficiency of the LC-PCR for clinical diagnosis of leprosy was further evaluated in a set of skin biopsy specimens. If the fluorescence signal did not increase within 45 cycles (C_T 45), the samples were considered negative. A negative template control, which included all reaction components except DNA template, was included in each run to detect carryover contamination. A few number of amplicons obtained from leprosy patients' samples were sequenced at core facilities at the National Institute of Health, Thailand, using the BigDye 3.1 ready reaction and the ABI Prism 310XL DNA sequencer (Applied Biosystems, Weiterstadt, Germany).

In order to establish a quantitative LC-PCR, standard curves that depicted a relation between the crossing point values, cycle numbers for the first significant increases in the fluorescence above the threshold level [C_T] and the logarithmic concentrations of *M. leprae* DNA, or the logarithmic numbers of leprosy bacilli, were constructed. The DNA standard curves were generated from two series of 10-fold dilutions of *M. leprae* DNA ranging from 20.0 to 0.002 ng and from 2×10^3 to 2.0 fg. Based on the DNA standard curve, the slope of a linear relation as calculated by the LIGHTCYCLER operation software was converted into the amplification efficiency using the formula $E = 10^{-1/\text{slope}} - 1$. A standard curve composed of DNA prepared from serial dilutions of known cell numbers of *M. leprae* was used for quantification of *M. leprae* in clinical samples. By normalization, the initial levels of nucleic acids in the reactions could be established based on the cycle threshold and, hence, the number of bacilli could be inferred. Enumeration of *M. leprae* cells in 10 skin samples randomly selected from the same group of these untreated multibacillary patients was also conducted by microscopic examination and then calculated for the actual number of bacilli in each specimen. The numbers of bacillary cells were compared with those quantitated using LC-PCR assay.

A linear regression of the standard curves of *M. leprae* DNA or *M. leprae* cell number vs. C_T values were automatically calculated by the quantitative mode of LC software. Difference between means of C_T values of multibacillary and paucibacillary groups was analyzed by Student's *t* test using the online GRAPHPAD software. Using functions in Microsoft Excel, the correlation of BI and logarithm of *M. leprae* cells quantitated using LC-PCR and the correlation of logarithm of *M. leprae* cells detected using LC-PCR and microscopy could be calculated.

Results

Rapid LC-PCR and continuous monitoring of the amplified product

The facile method for extraction of DNA from skin tissue homogenates allowed successful amplification using LC-PCR. The amplification plot and melting curve profile performed in the analysis of the sensitivity of the test are shown in Fig. 1a and b, respectively. The SYBR Green generated a fluorescence signal and fluorescence acquisition occurred within the F1 channel. The fluorescence detection was achieved during the PCR cycle running in real time. To achieve a good fluorescence signal, the $MgCl_2$ concentration was increased up to 4 mM. The exponential nature of the amplification curve indicated optimal PCR and the crossing point corresponding to cycle number when the exponential phase begins could be identified. The signal started to increase at different times depending on the concentration of DNA templates (Figs 1a and 2a). The fluorescence signal results in a characteristic sigmoidal-shaped curve,

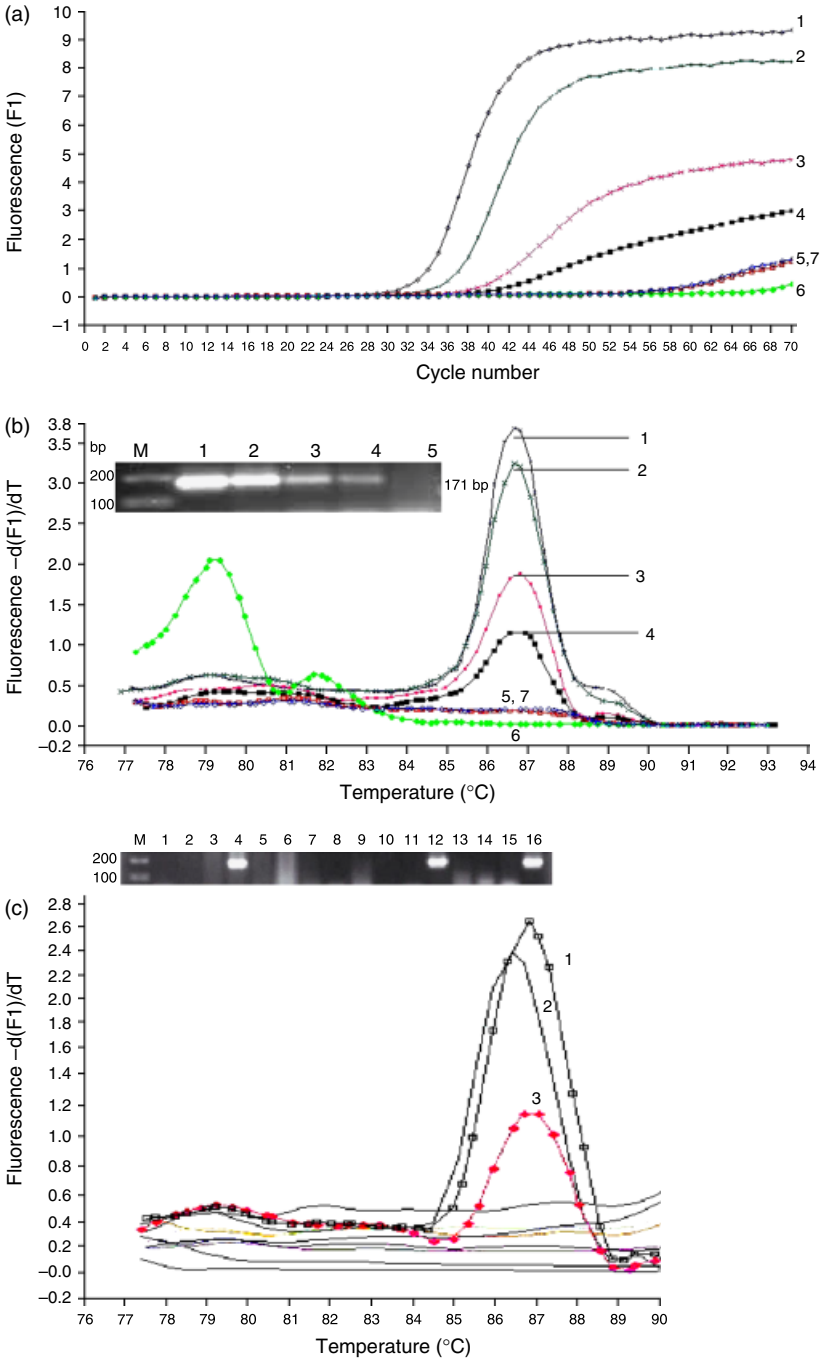

Fig. 1. Sensitivity and specificity of the test. (a) Amplification curves of LC-PCR in analysis of the detection limits of *Mycobacterium leprae* DNA. The sensitivity of LC-PCR for the detection of *M. leprae* DNA was evaluated with various DNA concentrations. Ten-fold dilutions of purified *M. leprae* DNA in the range of 20 pg–0.2 fg were prepared and subjected to LC-PCR amplification. The fluorescence signal was detected in real time. Lines: 1, 20 pg; 2, 2.0 pg; 3, 0.2 pg (200 fg); 4, 0.02 pg (20 fg); 5, 0.002 pg (2.0 fg); 6, 0.0002 pg (0.2 fg); 7, negative control. (b) Melting peaks of LC-PCR products and agarose gel electrophoresis in analysis of the detection limits of *M. leprae* DNA. Amplicons obtained from LC-PCR amplification using serial dilution of purified *M. leprae* DNA were identified for 171-bp fragment of *M. leprae* 16S rRNA gene by melting curve analysis. Lines: 1, 20 pg; 2, 2.0 pg; 3, 0.2 pg (200 fg); 4, 0.02 pg (20 fg); 5, 0.002 (2.0 fg); 6, 0.0002 pg (0.2 fg); 7, negative control. Gel electrophoresis verified detectable fluorescence signals in melting curves analysis. Lanes: M, DNA marker; 1–5, samples corresponding to lines 1–5. (c) Specificity of LC-PCR for *M. leprae*. DNA was extracted from mycobacteria that may cross react with *M. leprae*. DNA from selected nonmycobacteria were also included and subjected to LC-PCR. Spiking *M. leprae* DNA was performed in a few reactions to determine reaction inhibition and to confirm the specificity of LC-PCR. Amplicons were identified for *M. leprae* DNA by melting curve analysis. Lines: 1, *Salmonella typhimurium* plus *M. leprae*; 2, *Mycobacterium tuberculosis* plus *M. leprae*; 3, *M. leprae*. Agarose gel electrophoresis of LC-PCR products verified specificity for *M. leprae*. Lanes: M, DNA marker; 1, negative control; 2, *Bacillus cereus*; 3, *S. typhimurium*; 4, *S. typhimurium* plus *M. leprae*; 5, *Escherichia coli*; 6, *Pseudomonas aeruginosa*; 7, *Streptococcus agalactiae*; 8, *Corynebacterium ulcerans*; 9, *Listeria monocytogenes*; 10, *Staphylococcus aureus*; 11, *Mycobacterium tuberculosis*; 12, *M. tuberculosis* plus *M. leprae*; 13, *Mycobacterium smegmatis*; 14, *Mycobacterium avium*; 15, *Mycobacterium bovis*; 16, *M. leprae*.

representing the three phases of PCR: the lag phase (little product accumulation), the exponential phase (rapid product accumulation) and the plateau phase (no further product is amplified). The amplified products were rapidly characterized postamplification by melting curve analysis. As expected, the amplicon size of the 171-bp fragment could be confirmed and was observed using agarose gel electrophoresis (Fig. 1b).

Sensitivity and specificity of LC-PCR for the detection of *M. leprae*

The analytical sensitivity of LC-PCR in detecting *M. leprae* was defined as the lowest amount of *M. leprae* DNA or the minimal number of bacilli detectable using LC-PCR. Using serial quantified DNA samples, this assay could accurately detect as low as 20 fg of DNA of *M. leprae* (Fig. 1a and b).

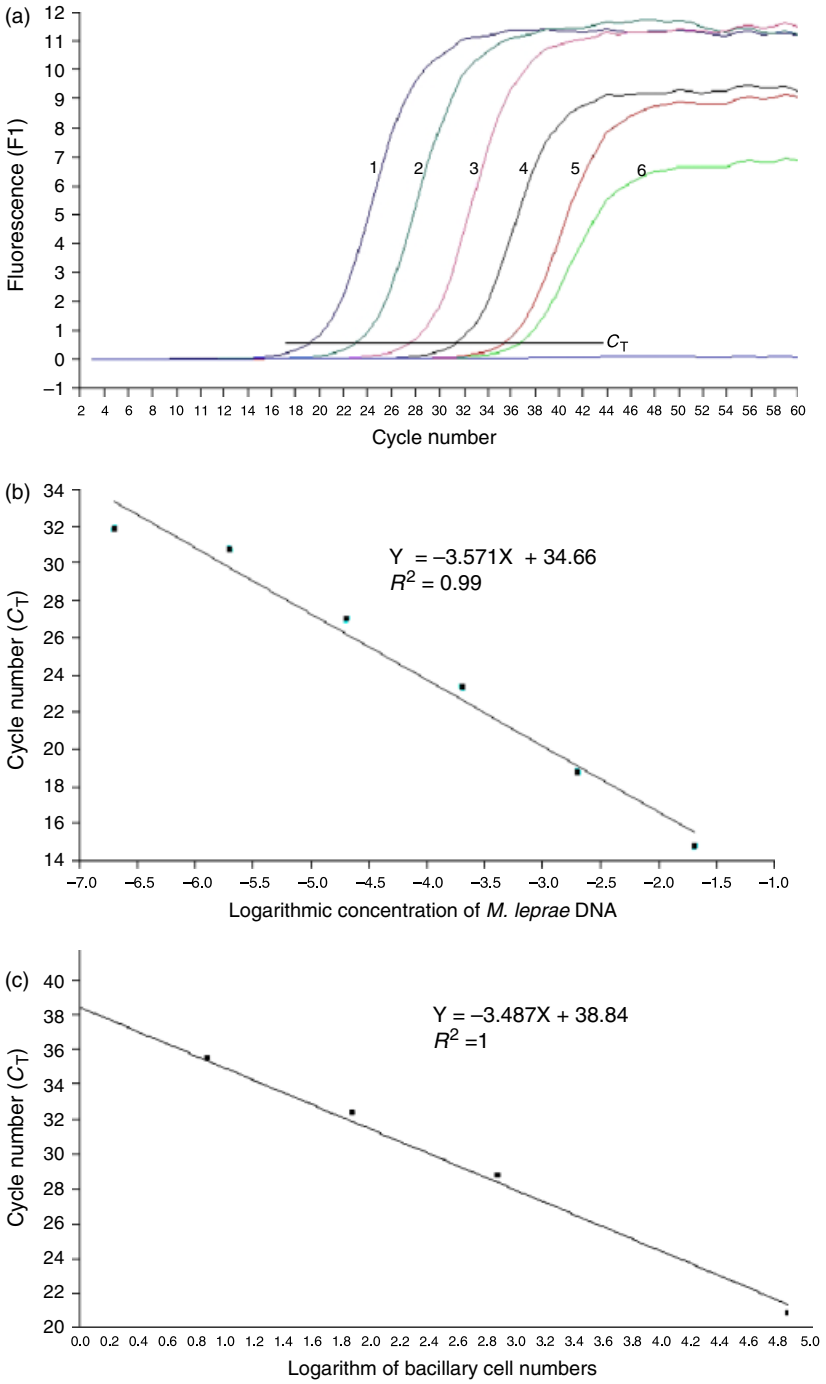

Fig. 2. Quantitative detection of *Mycobacterium leprae* DNA using LC-PCR. (a) Analysis of crossing points of cycle threshold (C_T). Ten-fold serial dilutions from 20.0 to 0.002 ng of purified *M. leprae* DNA were prepared and subjected to LC-PCR. Crossing points in LC-amplification curves were analyzed to create a standard curve in LC-PCR quantitative assay. (b) A linear regression of *M. leprae* DNA standard curve. Serial 10-fold dilutions from 20.0 to 0.002 ng of *M. leprae* DNA were amplified and analyzed using LC-PCR. The relation of serial dilutions of *M. leprae* DNA and fluorescence signals at crossing points was constructed. The standard curve displayed a linear regression of the threshold cycles (C_T) vs. the logarithm of the initial concentrations of *M. leprae* DNA (log[DNA]) in LC-PCR reactions. (c) A linear range of *M. leprae* standard curve. *M. leprae* DNA was extracted from dilutions containing 200 000, 2000, 200, 20 and 2 *M. leprae* cells and subjected to LC-PCR. The *M. leprae* cell standard curve was generated by a linear regression of the threshold cycles (C_T) vs. the logarithm of leprosy bacilli.

Mycobacterium leprae DNA extracted from leprosy skin specimens containing 2×10^3, 200, 20 and two bacilli was also detected using LC-PCR supporting the high sensitivity of the assay. By melting curve analysis, a single amplicon, giving a distinct melting temperature (T_m of $86.5 \pm 1.0\,^{\circ}C$), could be identified only when the reactions contained *M. leprae* DNA (Fig. 1b and c). The negative controls produced no amplification signals. Sequencing of amplified products from patients verified the 171 bp of DNA fragment as corresponding to a portion of 16S rRNA gene specific to *M. leprae*.

Clinical efficiency of LC-PCR

Application of LC-PCR to skin specimens was evaluated by comparing LC-PCR results with the clinical diagnosis of 66

leprosy patients. Excised tissues from skin lesions of clinically diagnosed leprosy patients were processed for *M. leprae* DNA. The 171-bp fragment of the *M. leprae* 16S rRNA gene was amplified using LC-PCR and the detectable fluorescence signal was analyzed by melting temperature and product detection was further confirmed using electrophoresis.

In the multibacillary cases, 38 of 38 biopsy specimens and 14 of 28 of the paucibacillary skin biopsies were positive by LC-PCR. Therefore, the overall sensitivity of LC-PCR was 100% in multibacillary and 50% in paucibacillary patients (Table 1). The analysis from the two-by-two contingency table indicated 100% specificity and 78.79% sensitivity for leprosy detection. All the positive samples were also positive by conventional PCR. Overall, the total running time, including DNA extraction and LC-PCR analysis, was *c.* 3 h.

Quantitative LC-PCR

Quantitation of *M. leprae* DNA was achieved using LC-PCR. The initial levels of nucleic acids could be determined based on the crossing point of the cycle threshold, the cycle number at which the first significant increase in the fluorescence above the threshold level is detected (Fig. 2a). The standard curves were automatically generated from the LC-PCR system by plotting cycle number (C_T) vs. either the logarithmic concentrations of standard *M. leprae* DNA or the logarithm of *M. leprae* cell numbers. A standard curve of diluted DNA for quantitative assay was linear, with a coefficient of determination (R^2) of 0.99 and a slope of -3.571 (Fig. 2b). Based on the slope of the regression of DNA standard curve analyzed using the LC software, the amplification efficiency of this quantitative LC-PCR was 92%. When DNA was extracted from serial dilutions of known cell numbers of *M. leprae*, a broad-range linear relationship between fluorescence signal at crossing points and cell number was also observed. Figure 2c shows a linear regression of a standard curve of *M. leprae* cells corresponding to a coefficient of determination (R^2) of 1 and a slope of -3.487 with a limit of detection of two leprosy bacilli.

The bacterial load of *M. leprae* could be quantified by interpolation based on a standard curve. Using the maximum option of the LC software for quantitative analysis, the number of *M. leprae* bacilli detected in a 6-mm^3 skin biopsy specimen from leprosy patients was in the range of 1.07×10^2–1.65×10^8 bacilli, with C_T values ranging from 16.98 to 41.72 cycles. Figure 3 shows the scattering plot of logarithmic cell numbers of leprosy bacilli vs. BI. It should be noted that the C_T values of paucibacillary patients were higher than those of multibacillary patients (mean $\pm$ SD of C_T for paucibacillary and multibacillary were 35.91 ± 3.76 and 25.86 ± 7.30, respectively). Comparison of C_T values in the two groups demonstrated a statistically significant difference ($P < 0.0001$). As expected, high C_T values were associated with low BI values, and low C_T values were associated with high BI. The numbers of *M. leprae* cells in slit skin smears were enumerated and reported as BI, which could be correlated to those in biopsy specimens quantitated using LC-PCR. The relation between BI (X-axis) and logarithmic cell numbers of *M. leprae* (Y-axis) in multibacillary patients was $Y = 0.475X + 3.607$, with a coefficient correlation (r) of 0.608. The number of *M. leprae* cells in 6-mm^3 skin biopsy specimens of 10 of the multibacillary patients quantified by this method was compared with those analyzed by microscopic examination, a conventional reference technique for *M. leprae* bacterial load measurement. The numbers of bacillary cells quantified by the two methods are presented in Table 2. Further data analyses showed the correlation of logarithm of *M. leprae* cell numbers in skin biopsy specimens detected using LC-PCR and microscopy with a coefficient correlation (r) of 0.491 and a slope of 0.431 ($Y = 0.431X + 2.510$; X and

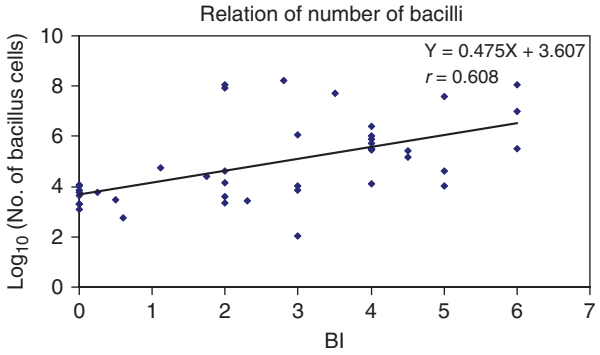

Fig. 3. Detection and quantitation of *Mycobacterium leprae* in clinical specimens. DNA was extracted from skin specimens and subjected to LC-PCR. Using the quantitative mode, the number of *M. leprae* bacilli were quantified by LC-PCR. The log $_{10}$ numbers of bacillary cells in 6-mm^3 skin specimens were plotted against the BI of individual patients.

Table 2. Comparison of the number of leprosy bacilli using quantitative real-time PCR and microscopic enumeration in 6-mm^3 skin biopsy punch specimens

No.	Leprosy type	BI	Microscopic enumeration (No. of bacilli)	Quantitative real-time PCR (No. of bacilli)
1	LL	6	2×10^7	3×10^5
2	LL	4	3×10^5	1×10^6
3	LL	4.5	1×10^4	1×10^5
4	LL	4	2×10^4	3×10^5
5	BL	2	7×10^2	4×10^4
6	BL	3	2×10^4	8×10^3
7	BL	4	2×10^7	3×10^6
8	BL	2	2×10^4	1×10^2
9	BL	5	7×10^4	1×10^4
10	BT	2.3	3×10^4	3×10^3

Y, cell numbers detected using microscopy and LC-PCR, respectively).

Discussion

The aim of this study was to develop and validate a real-time PCR assay that can rapidly detect *M. leprae* in clinical skin samples. We also set out to investigate the feasibility of using real-time PCR to accurately estimate the numbers of bacilli in skin specimens using predetermined standards.

The application of real-time PCR for rapid detection and quantification of *M. leprae* in clinical and experimental samples is attractive and should be the choice methods because microscopic enumeration of *M. leprae* has severe limitations. The present study describes a simple and sensitive real-time PCR assay using the LightCycler™ and SYBR Green for quantitative detection of *M. leprae*. When DNA extraction was used in conjunction with LC-PCR, results were obtained in < 3 h. LC-PCR detection increased the speed of PCR diagnostics by rapid thermal cycling and by replacing gel electrophoresis with rapid melting curve analysis. The specificity of primers used was examined extensively in previous studies assessing them against a variety of nonmycobacteria and mycobacteria that might cross react with *M. leprae* (Cox *et al*., 1991; Kurabachew *et al*., 1998; Phetsuksiri *et al*., 2006). Using highly characterized and specific primers, the LC-PCR assay showed 100% specificity for *M. leprae* and showed no interference by overloading with nonspecific nucleic acids when skin samples were tested. The sensitivity achieved was such as to detect as low as 20 fg of purified *M. leprae* DNA. Estimations of the number of equivalent bacilli were based on the fact that *c.* 5 fg of DNA corresponds to one bacterium (Clark-Curtiss *et al*., 1985). By this estimation, the minimal number of *M. leprae* that this assay could detect is four bacilli. This calculated number was very close to the minimal number of two bacilli that LC-PCR was able to detect experimentally, as described above.

Quantitation of DNA from microorganisms using real-time PCR can be conducted based on the crossing point for each sample. This crossing point or threshold is unique for a particular concentration of DNA and can allow construction of a standard curve providing a means to determine the corresponding concentrations of unknown samples. The crossing points of standards were plotted and used to quantify the amounts of DNA targets or the number of organisms in unknown samples. In this study, it was demonstrated that the LC-PCR assay facilitated specific quantification of *M. leprae* in skin biopsy specimens over a range of 1.07×10^2–1.65×10^8 cells per skin tissue samples, and the C_T values of paucibacillary were higher than those of multibacillary patients. This makes for a promising method that can complement standard means for classification of leprosy patients. The assay was able to confirm leprosy diagnosis in 100% of multibacillary and 50% of paucibacillary patients indicating practical sensitivity, a considerable improvement on earlier efforts (Kramme *et al*., 2004). The results of the application of LC-PCR in skin specimens also suggested that the efficiency of LC-PCR for leprosy diagnosis was superior to that of skin smear microscopy because 50% of paucibacillary (microscopically negative) patients in this study were LC-PCR positive. The positive detection of *M. leprae* by skin smear microscopy requires 10^4 leprosy bacilli g^{-1} of tissue (Shepard & McRae, 1968). Based on the assumption that the 6-mm^3 skin biopsy specimen weighted 10 mg as described previously (Martinez *et al*., 2006), it was concluded that at least 100 mycobacteria must be present in order to be able to detect *M. leprae* in this kind of specimen by microscopy. Considering the input with our LC-PCR threshold (four bacilli), it was proposed that this real-time PCR method is at least 25 times more sensitive than microscopic examination for AFB. The LC-PCR positive rate in the paucibacillary group was lower than that of multibacillary patients due to the lower number of bacilli present. Despite the low positive rate in paucibacillary patients, the ability of LC-PCR to detect *M. leprae* DNA using LC-PCR on regular bacteriological negative samples would be helpful in differentiating leprosy from diseases that cause similar symptoms, ensuring a correct diagnosis.

The application of the assay in particular to difficult-to-diagnose cases such as in patients with single lesion or with no detectable bacilli may be very useful. As expected, positive results by microscopic examination of slit skin smear perfectly matched with positive LC-PCR results and the higher BI were detected earlier in cycling amplification and in demonstrating low C_T. It was demonstrated that specimens from high BI patients appeared to have high numbers of bacteria as detected using LC-PCR. LC-PCR is now proposed as an alternative method for the detection and quantitation of *M. leprae* and it can be applied to clinical specimens to aid in enumeration of *M. leprae*. The application of LC-PCR may be limited to a few reference centers according to the availability of machines, technical demand and monetary reasons. It is more costly than microscopy. However, because nowadays many public health laboratories are using real-time PCR for the detection of a range of pathogens due to its flexibility, speed, real-time advantage and high sensitivity, it is feasible to adapt and establish this assay in such settings. Also, it is applicable and useful for research purposes in experimental quantification of *M. leprae*.

In conclusion, this LC-PCR method is a sensitive, specific and facile means for the rapid detection of *M. leprae* in skin specimens. The assay provides an alternative to classical acid-fast staining. Based on the fact that no culture system exists for *M. leprae*, a particularly useful application of this

assay is to accurately and rapidly quantitate the number of bacilli as a gauge for the appropriate management of leprosy patients.

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