Quantitative real-time PCR analysis of *Mycobacterium leprae* DNA and mRNA in human biopsy material from leprosy and reactional cases

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Mycobacterium leprae, the causative agent of leprosy, is uncultivable in defined media. Development of new diagnostic tools which do not depend on growth of bacteria is needed for the early detection of *M. leprae* and for monitoring the effectiveness of chemotherapy. We used a real-time PCR-based assay to quantify the copy number of bacterial DNA and *hsp18* mRNA from 47 leprosy patients using paraffin-embedded biopsy samples. The assay used was specific, sensitive and reproducible. The applicability of this approach in monitoring the chemotherapy of leprosy was examined. A reduction in DNA and mRNA during chemotherapy was observed and *hsp18* mRNA could not be detected in patients who underwent 2 years of multidrug therapy (MDT). However, a considerable amount of *M. leprae* DNA could be detected even after 2 years of MDT. A significant amount of *hsp18* mRNA was found in reactional cases as well. This raises important questions regarding the role of bacterial antigens in leprosy reactions and the rationale of omitting antibiotics in the treatment of reactional cases. Results in this study show that real-time PCR could be a better tool for the careful monitoring of bacillary DNA and mRNA in lesions, which will help to improve diagnosis, disease progression and the treatment regimen.

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INTRODUCTION

Leprosy is a chronic granulomatous infection of the skin and peripheral nerves caused by *Mycobacterium leprae*, an uncultivable bacterium (Britton & Lockwood, 2004). Leprosy is a slowly progressive, spectral disease. At one end of the spectrum is tuberculoid leprosy wherein patients mount a strong cell-mediated immune response against *M. leprae*, resulting in the reduction and eventual clearance of the infecting bacteria. At the other end of the spectrum is the lepromatous condition, in which patients display disseminated infection with high bacillary load, high levels of anti-*M. leprae* antibodies, and a weak cell-mediated immune response towards *M. leprae* antigens. In-between these two polar forms, unstable borderline cases with specific clinical, immunological and pathological characteristics exist (Ridley & Jopling, 1966). A significant fraction of borderline patients undergo immunological reactions called type 1 (reversal) and type 2 (erythema nodosum leprosum or ENL) (Gupta *et al.*, 2005; Scollard

et al., 2006). Despite a marked reduction in the prevalence of leprosy since the implementation of multidrug therapy (MDT), the detection rate of new cases has not shown appreciable decline (Britton & Lockwood, 2004; Naafs, 2000). Standard immunological and histological approaches for assessing leprosy have been shown to be less effective in the diagnosis of early leprosy. In addition, there are no satisfactory methods for monitoring the efficacy of treatment in the patients undergoing reversal reactions (Katoch, 2002). Acid-fast staining and PCR-based approaches have been widely used for diagnosis, but the sensitivity and specificity of both these methods is limited (Job *et al.*, 1991; Naafs, 2000; Plikaytis *et al.*, 1990; Williams *et al.*, 1990). Recently, real-time PCR-based methods have been developed for the diagnosis of leprosy by DNA-based quantification, but the clinical sensitivity has not been shown to be higher than that of conventional PCR (Kramme *et al.*, 2004; Martinez *et al.*, 2006).

Formalin-fixed and paraffin-embedded tissue (PET) is less vulnerable to contamination for molecular pathology studies and diagnostic applications (Santhosh *et al.*, 2005; Shabaana *et al.*, 2001). The use of PET in bacterial load determination and gene expression studies by real-time PCR has been reported to be very sensitive and highly

Abbreviations: ENL, erythema nodosum leprosum; MDT, multidrug therapy; PET, paraffin-embedded tissue.

Supplementary information is available with the online version of this paper.

reproducible (Godfrey *et al.*, 2000; Lehmann & Kreipe, 2001). Several *M. leprae*-specific genes have been used as targets in the diagnosis and treatment of leprosy (Cox *et al.*, 1991; Donoghue *et al.*, 2001; Jamil *et al.*, 1993; Martinez *et al.*, 2006; Rastogi *et al.*, 1999). Earlier reports show that *hsp18* can be used as an effective marker for the early detection of *M. leprae* and for analysing the effect of MDT (Chae *et al.*, 2002). A more sensitive and rapid method for the detection of *M. leprae* and its gene expression analysis would add an unbiased criterion to the available means of diagnosis and assessing the validation of the effectiveness of chemotherapy.

In this study, we have used PET for the examination of the DNA and mRNA of the *hsp18* gene in various stages of leprosy. We have analysed the change in expression of *hsp18* DNA and mRNA in leprosy and reversal reactions and also validated the usefulness of this method in monitoring the chemotherapy of leprosy. To our knowledge, this is the first report analysing the effectiveness of real-time PCR in quantifying bacterial RNA and DNA in PET.

METHODS

Patients and samples. Leprosy patients were classified according to Ridley & Jopling (1966). Punch biopsies from patients were collected after obtaining informed consent, as per the standards laid down by the institutional ethical committee and Indian Council of Medical Research. This study included 47 patients across the leprosy spectrum: tuberculoid ($n=6$), borderline tuberculoid ($n=9$), borderline lepromatous ($n=8$), lepromatous ($n=9$), with a type I reaction or reversal reaction ($n=7$) and with a type II reaction or ENL ($n=8$). Details of the patients used in this study are shown in Supplementary Table S1 in JMM Online. The bacterial load in each biopsy sample was determined by acid-fast staining and expressed on a logarithmic scale as the bacterial index.

Extraction of DNA from PET. Biopsy materials were fixed in formaldehyde and embedded in wax (Shabaana *et al.*, 2001). Thirty, 10 μm sections were collected in an Eppendorf tube and deparaffinated using xylene. The pellet was washed with 70% ethanol and dried under vacuum. One millilitre of lysis buffer (10 mM EDTA, 100 mM NaCl, 150 mM Tris/HCl, 0.5% SDS) was added and sonicated in a sonicator bath (Labsonic System, Sonics and Materials) at 80% amplitude for 5 min. Lysozyme and proteinase K (Sigma) were added to a final concentration of 2 mg ml⁻¹ and 300 μg ml⁻¹, respectively. Samples were incubated for 6 h at 37 °C, and then treated with 1 μl RNase (10 μg μl^{-1}) for 20 min at 37 °C. Samples were boiled for 10 min and DNA was extracted using phenol:chloroform and precipitated using 2-propanol. DNA concentration was determined by measuring the A_{260} using a spectrophotometer (HU2000; Hitachi).

Extraction of RNA and preparation of cDNA. Total RNA from PET biopsies was extracted as described previously (Shabaana *et al.*, 2001). In brief, sections were deparaffinated in xylene and samples were incubated in digestion buffer (10 mM Tris/HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, proteinase K at a final concentration of 300 μg ml⁻¹) for 24 h at 52 °C followed by Trizol extraction. RNA was treated with DNase according to the manufacturer's instructions (Promega). The concentration of RNA was checked using a spectrophotometer. Total RNA (3 μg) was reverse-transcribed in a

final volume of 50 μl using a conventional programmable thermal cycler (MJ Research). The reaction mix containing RNA, 1 μg oligo dT₁₅ and 1 μg random hexamers (Promega) was incubated at 70 °C for 10 min and chilled on ice. Master mix containing 10 μl M-MLV reaction buffer (5 $\times$), 2.5 μl dNTP mix (10 mM each), 2 μl RNase inhibitor (20 U μl^{-1}) and 200 U M-MLV reverse transcriptase (Amersham Pharmacia Biotech) was added and incubated at 37 °C for 1 h. The enzyme was heat-inactivated at 92 °C for 2 min and cDNA was stored at -20 °C. Assay without M-MLV reverse transcriptase was included as a negative control for each sample analysed (reverse transcriptase-minus controls). Total DNA extracted from psoriasis patient biopsy samples was used as a negative control (see Supplementary Fig. S1 in JMM Online).

Conventional PCR. PCR amplification of the template DNA and RNA was carried out using thermal cycler PTC 200 (MJ Research). Cycling parameters were: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min for 36 cycles, followed by a final extension for 7 min. PCR was performed in a 25 μl reaction mix containing 5 μl cDNA, 1.25 U *Taq* polymerase (Fermentas), 2 μl MgCl₂ (final concentration 1 mM) and 2 μl dNTPs (final concentration 100 μM). PCR products were electrophoresed in an agarose gel and visualized by ethidium bromide staining.

Real-time PCR analysis. Real-time PCR analysis was performed using SYBR Green chemistry in an ABI PRISM 7000 Sequence Detection system (Applied Biosystems). All the primers were designed using Primer Express Software (Applied Biosystems) and the primers were purchased from Microsynth. Oligonucleotide sequences of the primers used for real-time PCR assays targeting the *hsp18* gene were: *hsp18F*, 5'-TTCGCCGAGCAAGTGTTAGG-3'; and *hsp18R*, 5'-GGTGACTACGTTGCGTTCGA-3'. Those used for the β -actin gene were: βF , 5'-CCACACTGTGCCCATCTAC-3'; and βR , 5'-AGGATCTTCATGAGGTAGCAGTC-3'. Two-step SYBR Green assays were performed in a reaction volume of 25 μl using SYBR Green Master Mix (Eurogentec). Primer concentrations were optimized using 50–900 nM; the optimum concentration was found to be 900 nM. Real-time PCR analysis was performed using the following optimized assay conditions: 10 min at 94 °C followed by 15 s at 94 °C and 1 min at 60 °C for 40 cycles. Amplifications were performed in duplicate or in triplicate wells. For each sample analysed, reverse transcriptase-minus controls and non-template controls were included. Melting curve analysis (Applied Biosystems software) was performed after each run to confirm the specificity of the primers used.

Bacillary load determination in leprosy biopsies. Segments of the genes encoding *hsp18* (amplicon size of 150 bp) and β -actin (amplicon size of 99 bp) were amplified from biopsy material using the same primers used for real-time PCR assay and cloned in pGEMT vector. The recombinant clones were confirmed by restriction digestion and automated DNA sequencing (ABI PRISM 377; Applied Biosystems). Copy numbers of the recombinant plasmids pLKr18 and pLKr β were calculated (Giulietti *et al.*, 2001). Single copy to 10⁶ copies of the plasmid DNA were used for standard curve preparation. Copy numbers of the DNA in biopsy samples were calculated from the above standard curve (Giulietti *et al.*, 2001), and expressed as copy number (μg total DNA)⁻¹. Absolute copy numbers of *hsp18* mRNA and β -actin mRNA were calculated using *hsp18* standard and β -actin standard, respectively. In order to normalize the reverse transcription efficiency and to compare the copy numbers in different samples, the copy number of *hsp18* mRNA was normalized against 1 $\times$ 10⁶ copies of β -actin mRNA, which corresponds to the copy number of *hsp18* mRNA (μg total RNA)⁻¹.

Inter- and intra-assay variation. Inter-assay variations were examined by running three independent sets of experiments done at different times. Intra-assay variations were also examined using copy numbers ranging from 10^7 to 10^2 on the same day using different stock dilutions of the same plasmid used for inter-assay variation in a single plate. The coefficient of variation was calculated for each assay (Klee *et al.*, 2006).

Statistical analysis. An ANOVA test was used to analyse the significant level of DNA copy number or mRNA copy number across the spectrum of leprosy. A difference with a *P*-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Specificity and reproducibility of the real-time PCR assay

In the present study, we used an integrated approach of quantifying bacteria by DNA analysis and mRNA-based quantification, using real-time PCR. In addition, we used paraffin-embedded leprosy biopsies for these studies, which reduce the chances of the direct handling of infectious material. The use of PET biopsy samples for the analysis of host- as well as pathogen-specific mRNA by RT-PCR has been reported in previous publications from our laboratory (Santhosh *et al.*, 2005; Shabaana *et al.*, 2001, 2003). Recombinant plasmid carrying the cloned amplicon, prepared using the same primer set used for real-time PCR analysis, was constructed, and used for the preparation of a standard curve. The standard curve obtained was linear over 6 logs with a correlation coefficient of 0.99 and amplification efficiency of 100 % (Fig. 1). The detection

limit of the assay was determined using serial dilutions of the recombinant plasmid pLKr18 as template and copy number was calculated using the standard graph. The same experimental conditions were used for the detection of *M. leprae*-specific genes. Results indicated that even a single copy was within the linear range of the curve (data not shown). Absence of primer-specific amplification in negative controls (Supplementary Fig. S1 in JMM Online) confirmed the specificity of the real-time assay, and the absence of primer dimer formation in melting curve analysis showed the specificity of the primers used (data not shown). We have used primers specific for the *hsp18* gene of *M. leprae* for quantification; the primers did not show any sequence similarity to *Mycobacterium tuberculosis* or *Mycobacterium avium* small heat-shock proteins. This gene has been shown to be useful in the analysis of the effect of MDT and can be used for strain typing of *M. leprae* (Chae *et al.*, 2002; Shabaana *et al.*, 2003). In addition to this, sequencing of the cloned amplicons in recombinant plasmid confirmed the specificity of detection of *M. leprae*. In order to assess the reproducibility of the assay, intra- and inter-assay variations were determined using the same recombinant plasmid as template (Klee *et al.*, 2006). The intra-assay coefficient of variation between two independent dilutions in the same run was 1.32, 0.52, 0.56, 0.78, 0.61 and 0.56 % with a mean coefficient of variation of 0.72 %. The inter-assay coefficient of variation between three independent runs was 0.55, 0.152, 0.44, 0.22, 1.44 and 0.72 % with a mean coefficient of variation of 0.58 %. These results show that the assay used is specific, sensitive and reproducible, with a detection limit of one bacillus per sample.

Bacterial load in leprosy lesions across the spectrum

M. leprae genome data showed the presence of a single copy of the *hsp18* gene per chromosome (Cole *et al.*, 2001). Detection of *hsp18*-specific DNA perhaps indicates the bacillary load in leprosy lesions. We have developed a simple method of DNA extraction from PET tissues which is simpler and safer than the earlier reported methods (Clark-Curtiss *et al.*, 1985). Disruption of tissues by cup-horn sonication reduced the possibility of contamination. An additional phenol:chloroform extraction increased the purity of DNA for real-time PCR assays. The copy number of *M. leprae* DNA in each sample was calculated by the absolute quantification method. *hsp18*-specific DNA could be detected in all cases analysed. The highest bacillary load was seen in the lepromatous end of the spectrum and the lowest in the tuberculoid end of the spectrum (Table 1). Total RNA from biopsy samples was extracted as described in Methods and *hsp18* mRNA copy number was calculated in each sample. In order to reduce assay variation, the same amount of RNA and cDNA was used in all of the assays. Standard curves for *hsp18* and the β -actin gene were prepared using the recombinant plasmids as described earlier. The copy number of β -actin mRNA and *hsp18*

Fig. 1. Preparation of a standard curve for absolute quantification of *hsp18* and β -actin genes. For standard preparation, target genes amplified using the same set of experimental primers were cloned in pGEMT vector and plasmid DNA was extracted. Standard curves were obtained by plotting log copy number of recombinant clones carrying the target genes, ranging from 10^6 to single copies, against the C_t value of the respective dilutions. The correlation coefficient of the graph was >0.99 and the PCR efficiency of the amplicon was 100 % in all experiments, indicating a precise linear relation.

Table 1. Comparison of bacterial load across the spectrum

Total DNA and RNA were extracted from paraffin-embedded biopsies from leprosy patients and copy number was calculated as described in Methods. Copy number was expressed as log copy number (μg total DNA/RNA) $^{-1}$. Standard deviations were calculated in each case.

Sample no.	No. of samples used	Disease status	Copy number of DNA	Copy number of mRNA
1	9	Lepromatous leprosy	$6.9 \times 10^6 \pm 8.9 \times 10^6$	$1.8 \times 10^5 \pm 2.4 \times 10^5$
2	8	Borderline lepromatous	$8.1 \times 10^5 \pm 7.1 \times 10^5$	$6.2 \times 10^4 \pm 7.5 \times 10^4$
3	9	Borderline tuberculoid	$5.6 \times 10^5 \pm 6 \times 10^5$	$8 \times 10^3 \pm 1.1 \times 10^4$
4	6	Tuberculoid leprosy	$3.4 \times 10^3 \pm 5 \times 10^3$	$5 \times 10^2 \pm 5.2 \times 10^2$
5	7	Reversal reaction	$7.5 \times 10^5 \pm 6.5 \times 10^5$	$1.5 \times 10^4 \pm 1.3 \times 10^4$
6	8	Erythema nodosum leprosum	$1.1 \times 10^6 \pm 8.1 \times 10^6$	$8.5 \times 10^3 \pm 1.1 \times 10^4$

mRNA was calculated in each sample. It was observed that there was no significant variation in copy number of β -actin mRNA across the leprosy spectrum, and, therefore, we concluded that β -actin can be used as a suitable housekeeping gene for normalization (Fig. 2). A mean of 1×10^6 copies of β -actin mRNA was present (μg total RNA) $^{-1}$. mRNA of *hsp18* could be detected in all lepromatous and borderline cases. However, mRNA could not be detected in two cases of tuberculoid leprosy analysed. The copy number of *hsp18* mRNA was high in the lepromatous leprosy samples and decreased towards the tuberculoid leprosy end of the spectrum. The absolute copy number of bacterial DNA and mRNA was compared across the leprosy spectrum. Even though significant amounts of DNA and mRNA of *hsp18* could be detected, individual variation in copy number of mRNA was more towards borderline cases. Borderline cases are unstable and may migrate to either end of the spectrum. Earlier reports by Wakade & Shetty (2006) using the mouse foot pad system demonstrated the presence of viable bacilli in borderline leprosy lesions, even when the acid-fast bacilli detected were small in number. These results show that the

presence of *hsp18* mRNA in borderline cases may indicate the viability of the pathogen. Variation in copy number of *hsp18* DNA and mRNA across the leprosy spectrum and in reactional cases was analysed by ANOVA ($P < 0.05$ was considered significant). A significant variation in copy number of DNA ($P = 0.0088$) and mRNA ($P = 0.031$) was observed across the leprosy spectrum. This shows the spectral manifestation of the disease.

With the success of MDT in the treatment of leprosy, attention is now on the problem of reactions, which are the most significant issue in the management of patients. These reactional episodes manifest even without the presence of live bacilli. Published reports show that the frequency of reversal reaction at the time of diagnosis varies between 2.6 % and 28 % (Kumar *et al.*, 2004; Lienhardt & Fine, 1994; Van Brakel *et al.*, 1994). On the other hand, ENL reactions have been reported to occur in more than 50 % of lepromatous leprosy cases and in about 25 % of borderline lepromatous cases in the pre-MDT era (Kumar *et al.*, 2004; Lockwood, 1996). Different treatment regimens have been

Fig. 2. Estimation of copy numbers of β -actin mRNA across the leprosy spectrum. LL, Lepromatous leprosy; BL, borderline lepromatous; BT, borderline tuberculoid; TT, tuberculoid leprosy; RR, reversal reaction; ENL, erythema nodosum leprosum. Total RNA was extracted from paraffin-embedded biopsy specimens of 47 patients across the leprosy spectrum as described and cDNA was prepared. A real-time PCR assay was carried out using β -actin-specific primers. The copy number of β -actin mRNA was calculated using pLK β standard and expressed as copy number (μg total RNA) $^{-1}$.

proposed for controlling the severity of reactions; none have given effective results in controlling reactions (Britton & Lockwood, 2004; Tadesse *et al.*, 2006). Among the seven samples analysed from patients undergoing type 1 (reversal) reaction, the presence of bacilli could be detected in all cases. Even though a considerable amount of bacterial DNA could be detected in all the samples analysed from ENL cases, bacterial mRNA could not be detected in one case (Supplementary Table S1; sample number 41). In this patient where mRNA expression levels could not be detected, reaction developed 2 years after the completion of 2 years of MDT. This may possibly be a late reaction, which can occur even after 8 years from the start of MDT treatment (Kumar *et al.*, 2004). These data show that the method of detection of pathogen-specific DNA and mRNA will help to differentiate late reactions from relapse. Also, data from a patient who had developed a reaction after 18 months of MDT treatment (Supplementary Table S1; sample number 46) showed lower mRNA copy number when compared to untreated cases. However, the DNA copy number did not show much variation (data not shown). It may be the presence of DNA which causes the severity of reactions. The presence of a large amount of mRNA in reactional lesions indicates that the pathogen is viable. These findings raise a critical issue in reconsidering the therapy in reactional cases to eliminate the residual pathogen, which could be a better approach in controlling late reactions and relapses.

Quantification of *hsp18* DNA and mRNA during treatment

In order to validate the applicability of DNA- and mRNA-based quantification in assessing chemotherapy, we have compared the *hsp18* DNA and mRNA copy number in six different lepromatous leprosy patients during different periods of antibiotic treatment. Samples were collected from each patient before the treatment and during the treatment course. There was a marked reduction in *hsp18* mRNA detected at different time periods of chemotherapy, and complete disappearance of the transcript was observed after 2 years of treatment in two of the samples. However, as shown in Fig. 3, unlike mRNA, there was a considerable amount of *hsp18*-specific DNA detected even after 2 years of antibiotic treatment. Despite the small number of samples used, the correlation is clear enough to indicate that it takes at least 2 years to reduce the mRNA level significantly. These results substantiate earlier reports which showed the presence of *M. leprae* even after 2 years of MDT (Sharma *et al.*, 1999). Earlier experiments used the mouse foot pad assay for checking the viability of the pathogen (Gupta *et al.*, 1999, 2005). The time-consuming mouse foot pad assay for the detection of *M. leprae* viability can be replaced by the novel real-time PCR assay, which is more accurate and easy to perform. However, the presence of *hsp18* mRNA in reactional cases as well as during prolonged periods of antibiotic treatment raises the interesting question of whether this stable *hsp18* mRNA

Fig. 3. Determination of *hsp18* mRNA after MDT. Total DNA and total RNA from six lepromatous leprosy patients was collected before the MDT treatment started and after different time periods during treatment. Copy numbers of *hsp18* DNA and mRNA were calculated as earlier. The time point of the post-treatment stage (duration in months) at which the sample was collected from each patient is shown on the x-axis. Copy number of DNA and mRNA before treatment and after MDT treatment is shown on the y-axis. Samples from two different patients were analysed for *hsp18*-specific mRNA at 24 months post-treatment. No detectable RNA could be found at this point in both cases.

Table 2. Comparison of the sensitivity of real-time PCR and conventional PCR

The sensitivity of conventional and real-time PCR approaches was compared in all cases analysed using the same set of primers. Samples were grouped as smear-positive and smear-negative based on the bacterial index, according to the Ridley & Jopling (1966) scale as described in the text. The presence of transcript in each case was considered as positive for the reaction. PCR sensitivity in each case was calculated and compared.

	DNA		RNA	
	Conventional PCR	Real-time PCR	Conventional PCR	Real-time PCR
Smear-positive cases				
PCR +	29/29	29/29	25/29	28/29
PCR sensitivity	100 %	100 %	86.20 %	96.55 %
Smear-negative cases				
PCR +	17/18	18/18	11/18	16/18
PCR sensitivity	94.4 %	100 %	61.1 %	88.8 %

is coming from the active bacilli or from the bacilli which are dormant. There are several reports which show that dormant bacilli maintain viability for extended periods and they can be resistant to antimycobacterials (Hu *et al.*, 2000). Our previous report showed that Hsp18 can act as a molecular chaperone and was found to be localized in the cell membrane (Lini *et al.*, 2008). A homologue of *M. leprae hsp18* in *M. tuberculosis* was shown to be over-produced under stress conditions (Ohno *et al.*, 2003). These reports along with the data presented in this report suggest the involvement of *hsp18* in survival of the pathogen under stress conditions, which may account for the stability of mRNA even during prolonged periods of chemotherapy.

Clinical sensitivity of real-time PCR

Even though real-time PCR has been shown to improve the sensitivity of detection of many pathogens over conventional approaches, the same has not been reported for *M. leprae* (Kurabachew *et al.*, 1998). For analysing the clinical sensitivity of the real-time PCR approach in quantification of the *M. leprae* transcript, samples were classified as smear-positive and smear-negative cases based on bacterial index, according to Ridley & Jopling (1966). Among the 47 samples analysed, 29 were from smear-positive samples and 18 from smear-negative patients. Among the 29 smear-positive cases analysed, DNA could be detected in all the samples by PCR and real-time PCR (Table 2). However, the mRNA transcripts could be detected only in 25 samples using conventional PCR, whereas real-time PCR could detect mRNA in 28 cases. Among the 18 smear-negative cases analysed, 17 samples showed positive amplification for *M. leprae* DNA by conventional PCR (94.4 %) and all samples were positive (100 %) by real-time PCR. However, for *hsp18* mRNA, only 11 samples showed PCR positivity by the conventional approach (61.1 %); 16 samples were PCR-positive (88.8 %) by real-time PCR. These results show that DNA-based quantification did not increase the clinical sensitivity, agreeing with previous data (Kramme

et al., 2004). However, there was a significant increase in sensitivity when mRNA-based approaches were used, especially in smear-negative cases.

Our results clearly confirm the correlation of MDT and decline in the gene expression level, indicating the usefulness of this simpler and safer approach to monitor the disease progression in leprosy and the efficacy of chemotherapy. In addition, real-time analysis shows that there is a significant amount of mRNA for the *hsp18* gene, even in reactional cases, indicating that the treatment regimen of reactional cases should take into consideration the possibility of existence of live bacilli in reversal cases. This method can also be utilized for the detection of small numbers of bacilli from histological specimens.

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