Molecular Determination of *Mycobacterium leprae* Viability Using Real-Time PCR

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Abstract

Mycobacterium leprae, the etiologic agent of leprosy is non cultivable on axenic media. Therefore the viability of M. leprae for clinical or experimental applications is often unknown. To provide new tools for M. leprae viability determination, two quantitative reverse transcriptase-PCR (qRT-PCR) assays were developed and characterized. M. leprae sodA mRNA and 16S rRNA were used as RNA targets and M. leprae repetitive element (RLEP) DNA was used to determine relative bacterial numbers in the same purified bacterial preparations or from crude biological specimens. Results demonstrated that both assays were good predictors of M. leprae viability during short-term experiments (48 hr) involving rifampin-treatment in axenic medium, within rifampin-treated murine macrophages (MΦ) or within immune-activated MΦ. Moreover, these results strongly correlated with that of other M. leprae viability assays, including radiorespirometry-based and LIVE/DEAD® BacLight™ viability assays. The 16S rRNA/RLEP assay consistently identified the presence of M. leprae in eight multibacillary leprosy patient biopsies prior to multidrug therapy (MDT) and demonstrated a decline in viability during the course of MDT. In contrast, the sodA/RLEP assay was only able to detect presence of M. leprae in 25% of pre-treatment biopsies.

In conclusion, new tools for M. leprae viability determination were developed. The 16S rRNA/RLEP RT-PCR M. leprae viability assay should be useful for both short-term experimental purposes and for predicting M. leprae viability in biopsy specimens to monitor treatment efficacy, whereas the sodA/RLEP RT-PCR M. leprae viability assay should be limited to short-term experimental research purposes.
Introduction

Leprosy is a chronic infectious disease of skin and peripheral nerves and is of special concern because it can progress to peripheral neuropathy and permanent progressive deformity. 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 substantial decline (2). One explanation is that standard immunological and histological approaches for disease assessment are less effective in the diagnosis of early leprosy and, therefore, disease and transmission can progress. In addition, in vitro *M. leprae* viability assays such as those based on radiorespirometry (RR) and LIVE/DEAD® BacLight™ Fluorescent Bacterial Viability Assay require large quantities of bacteria $10^7$ and $10^6$, respectively, for reliable detection and therefore are not applicable for direct detection in clinical specimens (27, 14). The bacterial index (BI) is a long-established method for monitoring the patients’ responses to chemotherapy by giving an estimation of the number of acid fast bacilli (AFB) present in skin smears of lesions and other specific sites of the skin of leprosy patients. The BI range is 1 to 6 where 1 is least amount of bacilli detectable and 6 the most. However, the BI drops very slowly during treatment (1 BI/yr therapy) and in some cases the BI remains unchanged during and post treatment, making it difficult to determine drug efficacy or relapse of active infection (24).

The fluorescent-based and RR assays are both suitable for viability determination of a nude (nu/nu) mouse footpad-derived preparations of *M. leprae* to serve as inoculum for in vivo and in vitro experimental infection models because these preparations reproducibly contain very high levels of *M. leprae* ($10^9$/ml). However, the ability of theses assays to monitor the viability of *M. leprae* as an indicator of environmental influence, host cell responses in infected cultures, or in...
animal models, where only low numbers of bacteria are used, is very limited. Therefore, there is
great need for a rapid and sensitive viability assay for *M. leprae*.

To address this, we developed and characterized two quantitative reverse transcriptase-PCR (qRT-PCR) assays to provide new tools for determination of *Mycobacterium leprae* viability for clinical and experimental purposes. *M. leprae* *sodA* mRNA and 16S rRNA were used as the RNA targets for these assays and *M. leprae* repetitive element (RLEP) DNA was used to determine relative bacterial numbers in the same purified bacterial preparations or from crude biological specimens. The sensitivity and specificity of the assay was examined and their ability to detect the viability in MB leprosy patients’ biopsies prior to and during MDT was analyzed. Results demonstrated that both assays were good predictors of viability for experimental purposes such as during short-term (48 hr) rifampin treatment in axenic medium or within rifampin-treated murine macrophages (MΦ) or immune-activated MΦ. Analysis of human biopsies from multibacillary (MB) leprosy patients followed for up to 2 years after initiation of leprosy MDT demonstrated that the 16S rRNA/RLEP assay but not the *sodA/RLEP* consistently identified the presence of viable *M. leprae* in MB biopsies prior to MDT and 16S rRNA levels declined during MDT treatment.

**Materials and Methods**

*M. leprae*. *M. leprae* Thai-53, maintained in continuous serial passage in the hind foot pads of athymic nu/nu mice (Hsd: Athymic nu/nu, Harlan Inc., Indianapolis IN) were isolated approximately 6 months post infection as previously described (27) and acid-fast bacilli (AFB) counts were then determined by direct count according to the method of Shepard et al. (23). The viability of each preparation was determined in axenic medium by the oxidation of C\textsuperscript{14} palmitate using the Buddemeyer RR technique described below, but using the day 1 counts per minute.
(cpms) as an indicator of viability. The bacterial preparations were held overnight at 4°C pending quality control testing for contamination. Freshly harvested, highly viable bacilli (≥ 80%) were used within 24 hr of harvest.

Radiorespirometry. The metabolism of *M. leprae* was used as an indicator of viability by determining the oxidation of \(^{14}\)C-palmitic acid to \(^{14}\)CO\(_2\) using Buddemeyer RR as previously described (5). Briefly, 1x10\(^7\) *M. leprae* were suspended in 1.0 ml of BACTEC 7H12B medium (Becton Dickinson, San Jose, CA, USA) in a 5 ml glass vial with a loosen cap. This vial was inserted into a wide-mouth liquid scintillation vial lined with filter paper impregnated with NaOH, 2,5-diphenyloxazole (Sigma-Aldrich) and Concentrate I (Kodak, Chicago, IL, USA). Daily counts per minute (cpms) were recorded and day 7 cumulative cpms were used as an indicator of viability.

Fluorescent staining for quantification of bacterial viability. The membrane integrity of individual *M. leprae* (as an indicator of viability in a suspension) was determined using a LIVE/DEAD® BacLight™ Viability Kit (Molecular Probes, Eugene, OR, USA) as previously described (27). Briefly, *M. leprae* were washed twice (10,000 x g for 5 min) in sterile normal saline and incubated for 15 min at room temperature with a final concentration of 6 µM Syto9 and 30 µM Propidium Iodine (PI). The bacteria were washed twice in normal saline, the pellet was resuspended in 20 µl of 10% (v/v) glycerol in normal saline and 5 µl of the suspension was placed on a glass slide with a glass coverslip. The total number of bacteria and the number of dead bacteria were enumerated by direct counting of fluorescent green and red bacilli, respectively using a fluorescence microscope with the appropriate single bandpass filter sets. The excitation/emission maxima are 480 nm/500 nm for Syto9 and 490 nm/635 nm for PI. The number
of viable bacteria in a preparation was then determined by subtracting the number of dead (red) bacteria from the total number (green) of bacteria and the percent of viable bacilli in a preparation after treatment was determined by dividing the number of viable bacilli after treatment divided by that prior to treatment at the same time point.

**Rifampin treatment in axenic medium.** *M. leprae* were subjected to rifampin treatment in axenic medium using the following procedure. A stock solution of rifampin (Sigma-Aldrich, ST Louis, MO, USA) was made in DMSO (100 ng/ml) and filter sterilized. Aliquots of $1 \times 10^8$ purified nu/nu mouse-derived *M. leprae* were added to 5 ml 7H9 medium supplemented with OADC and containing 20 µg/ml final concentration of rifampin. Cultures were maintained at 33°C, optimum temperature for maintenance of *M. leprae* viability, (27) and 5% CO$_2$ for up to 2 wk. Controls consisted of *M. leprae* in 7H9 medium plus the same concentration of DMSO added to drug-treated *M. leprae*.

**Preparation and treatment of macrophage cultures.** RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine (Sigma-Aldrich) and 10% (v/v) FCS was used throughout these studies. Resident peritoneal cells from Swiss Webster mice (Harlan, Indianapolis, IN, USA) were harvested and allowed to adhere for at least 6 hr at 37°C and 5% (v/v) CO$_2$ on LUX plastic coverslips (Miles Laboratory, Elkhart, IN, USA) in 24-well tissue culture plates (Corning Incorporated, Corning, NY, USA) as previously described (19). After washing to remove non-adherent cells, the adherent macrophages (MΦ) were either infected with fresh nu/nu mouse-derived *M. leprae* at an MOI 20:1 for 16 hr at 33°C or first activated with 500 IU IFN-γ/ml (R& D Systems, Minneapolis, MN, USA) and 5 ng/ml LPS (Sigma-Aldrich) for 6 hr then infected with *M.
Extracellular \textit{M. leprae} were removed by washing the coverslips. Non-activated MФ were then treated with rifampin at 20 \(\mu\text{g/ml}\) (final concentration) or 50 \(\mu\text{g/ml}\) ampicillin (final concentration) (Sigma-Aldrich) and cultures were maintained for up to 48 hr. Non-treated infected cells served as controls.

**Patient skin biopsies.** A total of 19 skin biopsies (4 mm\(^3\) punch) and skin slit smears from eight multibacillary (MB) leprosy patients were obtained for leprosy diagnosis and follow up after initiation of MDT at the National Hansen’s Disease Programs Outpatient Clinic at Ochsner’s Hospital, Baton Rouge, LA. One half of each biopsy was formalin-fixed and paraffin-embedded using standard techniques. The remaining biopsy material was stored frozen in OCT preservative at -80\(^\circ\text{C}\) for 3-8 yr prior to use in this study. Patients were classified by clinical signs, BI in skin smears and histopathology of stained paraffin sections according to the Ridley Jopling scale (21). All patients in this study were classified with either borderline lepromatous leprosy (BL) or lepromatous leprosy (LL). Before the study was undertaken, it was reviewed by the LSU-Institutional Review Board (IRB), Baton Rouge, LA. Since the specimens were unused portions of skin biopsies taken for diagnostic purposes and coded so that names of individual patients were not available to the research staff or for any other purpose of the study, this study was determined to be exempt for the purpose of Human Subjects review.

**Extraction of \textit{M. leprae} RNA and DNA.** \textit{M. leprae} RNA and DNA were simultaneously extracted from the same sample using TRIzol\textsuperscript{®} reagent (Invitrogen) and a modification of the previously described single-tube homogenization/RNA extraction protocol using FastRNA\textsuperscript{®} Blue tubes (FastRNA\textsuperscript{TM} Kit-Blue, MP Biomedicals, Santa Ana, CA, USA), and a FastPrep\textsuperscript{®} FP 24 instrument (MP Biomedicals) (32). \textit{M. leprae} from axenic media were pelleted and washed x 2 in...
cold PBS at 14,000 x g, 4°C for 10 min and resuspended in 80 µl sterile DEPC-treated H$_2$O. For

*M. leprae* in frozen skin biopsies, the OCT was partially thawed on ice. The tissue (approximately

30 mg) was removed from the OCT, rinsed x 3 briefly in sterile cold PBS, and minced into small

pieces with a sterile scalpel in 200 µl TRIzol® reagent. For *M. leprae* in infected MΦ cultures,

MΦ were lysed with 0.1 N NaOH (750 µl) for 3 min then neutralized with an equal volume of 0.1

N HCl. Bacteria were pelleted (4°C) and washed x 2 in cold PBS. TRIzol® reagent was added to

all sample preparations to a total volume of 1 ml prior to adding to FastRNA® Blue tubes.

Samples were homogenized twice in the FastPrep® FP 24 instrument at a speed setting of 6.5 for

45 sec. Tubes were allowed to cool for 2 min between homogenizations. After homogenization,

tubes were chilled on ice for 5 min then 200 µl chloroform/isoamyl alcohol (24:1) were added and

tubes were mixed by vortex for 10 sec and then spun at 700 x g, 4°C for 5 min. The liquid was

transferred to a new tube an spun again at 14,000 x g, 10 min. *M. leprae* RNA was purified from

400 µl of the aqueous phase and DNA was removed from RNA preparations using the DNA-

free™ kit (Ambion, Inc., Austin, TX, USA) as specified by the manufacturer and precipitated

using standard techniques and resuspended in 30 ul DEPC-treated H$_2$O and stored at -80°C until

use.

DNA was purified from the remaining aqueous phase and interphase of the FastRNA® Blue tubes.

Briefly, 100 µl of 10 mM tris-EDTA pH 8.0 (TE) and 150 µl chloroform/isoamyl alcohol (24:1)

were added to the remaining aqueous phase and interphase material (~500 µl) and homogenized in

the FastPrep® FP 24 instrument twice. After centrifugation 14,000 x g, 10 min., the aqueous phase

was transferred to another tube and precipitated with 0.3 M NaOAc and 2 volumes of cold ethanol.
The DNA pellet was washed in ethanol 70% and dissolved in 30 µl of sterile, distilled water and stored at -80°C until use.

**Reverse transcription of *M. leprae* RNA.** RNA (500 ng) was converted to cDNA using the Advantage cDNA Polymerase Mix and Advantage RT-for-PCR Kit (BD Bio-Sciences) according to the manufacturer’s recommendations using Random Hexamer primers which are a mixture of oligonucleotides representing all possible sequences for a hexamer and included in the kit. Controls for DNA contamination consisted of total RNA incubated with the reverse transcription reagents excluding reverse transcriptase RT (-), human and mouse cDNAs.

**Real-time PCR.** The levels of *M. leprae* sodA mRNA and 16S rRNA in *M. leprae* from axenic medium, cultured MΦ, or in skin biopsy specimens were determined using real-time RT-PCR. These levels were normalized for bacterial numbers using a previously characterized, DNA-based, real-time PCR assay for the *M. leprae*-specific repetitive element (RLEP) (29). Primers and probes were designed using Primer Express 2.0 software, Applied BioSystems (Table 1). Purified *M. leprae* DNA or cDNA (5 µl) were added to a total PCR reaction volume of 25 µl containing TaqMan 2X master mix, 500 nM of each primer and 100 nM of each probe for RNA-based PCR assays (sodA mRNA or 16S rRNA) or 200 nM of each primer and 100 nM of the probe for the DNA-based PCR assay (RLEP). Reaction mixtures were subjected to 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min using a 7300 Real Time PCR System (Applied BioSystems, Carlsbad, CA, USA). A standard curve for each PCR assay was generated using serial 10-fold dilutions of purified *M. leprae* DNA ranging from 10 ng - 10 fg. Unknown values were interpolated automatically for each sample using the standard curve method and
normalized using the RLEP data for the same sample. The viability of *M. leprae* in human skin biopsies was determined by comparing normalized 16S rRNA values for patients prior to and up to 2 yr after start of MDT treatment.

**Specificity and sensitivity of assays.** The specificity of each real-time PCR TaqMan assay was determined by analyzing 10 ng of purified DNA from *M. leprae*, nine other mycobacterial species including: *M. tuberculosis* H37Rv ATCC 27294, *M. marinum* ATCC 927, *M. bovis* BCG ATCC 35734, *M. ulcers* ATCC 19423, *M. simiae* ATCC 25275, *M. avium* ATCC 25291, *M. intracellulare*, ATCC 13950, *M. kansasii* ATCC 35775, *M. smegmatis*, ATCC 14468; and other bacterial species including *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 12345, and *E. coli* ATCC 25992. In addition, mouse and human cDNA were also analyzed. The sensitivity or lower limit of detection of each assay was determined by analyzing 10-fold serial dilutions of *M. leprae* DNA and identifying the highest dilution, which still gave a positive value (Ct <37).

**Statistical Analysis.** The standard curves for each RT-PCR assay using Ct values versus serial DNA or cDNA concentration were calculated using a linear regression model (GraphPad InStat version 3 software). The means and standard deviation of the results of sodA/RLEP and 16S rRNA/RLEP RT-PCR assays for detection of *M. leprae* viability in axenic medium and MΦ cultures under various treatments were calculated using Student-t test. An alpha value of 0.05 was used for all analyses. All statistical comparisons were made using linear Pearson correlation coefficient (r) (GraphPad InStat version 3 software) as a measure of correlation between assays at a particular time interval.
Results

Identification of suitable targets for viability assays. Results of sodA/RLEP and 16S rRNA/RLEP RT-PCR assays demonstrated that levels of sodA gene transcripts were reduced to 5% that of non-treated controls 48 hr post rifampin treatment (Fig. 1A) and that these levels were significantly lower ($p < 0.001$) than that of 16S rRNA which were reduced to 38% of that of non-treated controls (Fig. 1B). However, after a week of treatment both were essentially background levels. In addition, reduction of sodA mRNA levels could be observed as early as within 12 hr of rifampin treatment compared to untreated controls (data not shown).

Specificity and sensitivity of molecular M. leprae viability assays. The specificity and lower limit of detection of each assay, sodA mRNA/RLEP and 16S rRNA/RLEP RT-PCR assays, were analyzed using DNA from nine other mycobacterial species, Staphylococcus epidermidis, Streptococcus pyogenes, E. coli and mouse and human DNA. Results showed that all three assays were resulted in values $Ct > 39$ indicating 100% specificity of these assays for M. leprae (data not shown). The sensitivity of detection of the M. leprae viability assays, defined as the lower limit of detection of M. leprae for each assay, was analyzed using serial 10-fold dilutions of purified M. leprae DNA and the standard curve method. The detection limit of sodA/RLEP assay was 1 pg ($Ct = 34.73 \pm 0.3$) and that of the 16S rRNA was 100 fg ($Ct = 36.42 \pm 0.6$). Since each M. leprae genome equivalent = 3 fg, these data suggest that these assays can detect 300 and 30 bacteria, respectively.

M. leprae viability determination in infected MΦ cultures. The ability of the RT-PCR-based M. leprae viability assays to detect the effects of anti-leprosy drug treatment in infected MΦ or the
effects of immune-activated murine MΦ (the host cell of \textit{M. leprae}) on \textit{M. leprae} viability was evaluated. The \textit{sodA}/RLEP RT-PCR assay results showed 16% viable \textit{M. leprae} remained in MΦ after 48 hr of rifampin treatment and only 1% viable \textit{M. leprae} remained after 48 hr infection of IFN-γ-activated MΦ (Table 2). Similar trends were noted for 16S rRNA/RLEP RT-PCR, LIVE/DEAD® BacLight™ Bacterial Viability Assay and radiospirometry assays. Thus, data from both molecular-based assays, 16S rRNA/RLEP and \textit{sodA}/RLEP, strongly correlated ($p < 0.0001$) with that from RR and LIVE/DEAD® BacLight™ Bacterial Viability Assay data (Table 2). However, the effects of the different treatments on \textit{M. leprae} viability were lower when compared to that of the \textit{sodA}/RLEP RT-PCR viability assay. Ampicillin-treatment led to a 20% decrease in \textit{M. leprae} viability using \textit{sodA}/RLEP RT-PCR assay, however, LIVE/DEAD® BacLight™ and RR both showed that ampicillin did not have an affect on \textit{M. leprae} viability. The 16S rRNA/RLEP showed that ampicillin has less of an effect on \textit{M. leprae} viability than that of the \textit{sodA}/RLEP assay. This result was expected since \textit{M. leprae} contains a β-lactamase enzyme which makes it resistant to ampicillin-like drugs (18).

\textbf{\textit{M. leprae} viability in paired MB leprosy patient skin biopsies.} A preliminary experiment was performed to determine the utility of the \textit{sodA}/RLEP and 16S rRNA/RLEP RT-PCR \textit{M. leprae} viability assays to detect \textit{M. leprae} viability directly from skin biopsy materials of MB leprosy patients. Results indicated that the \textit{sodA}/RLEP assay was only able to detect \textit{sodA} cDNA from two of eight pre-treatment biopsies (data not shown) and therefore the remaining biopsies taken after initiation of MDT from the same patients were not evaluated. In contrast, the 16S rRNA/RLEP assay showed positive results in all pretreatment specimens and declined during MDT treatment (Table 3). Moreover, when the BI of patients’ skin slit smears were compared to the number of \textit{M. leprae}...
leprae as a function of M. leprae DNA using real-time RLEP PCR, a significant association was observed ($r = 0.6942; p = 0.001$). Higher DNA concentrations correlated with higher BI values and lower DNA concentrations correlated with lower BIs (Fig. 2). However, when 16S rRNA/RLEP viability data were compared to patients’ BIs no correlation was observed ($r = 0.4604; p = 0.0842$).

Discussion

Determination of M. leprae viability is extremely difficult primarily due to the inability to cultivate this bacterium on axenic media. The discovery by Shepard in 1960 (23) of the mouse foot pad (MFP) technique to demonstrate replication of M. leprae was a research milestone, permitting testing of new anti-leprosy drugs, determination of drug-resistant strains of M. leprae from patient specimens and initial evaluation of vaccine protection. Variations of the MFP technique involving titration in large numbers of mice have been shown helpful to detect differences in the relative viability of different suspensions of M. leprae (30). However, this labor-intensive, time-consuming, expensive technique is impractical for the study of interactions of M. leprae with its host cell in vitro. Further complicating this is the declining availability of mouse foot pad laboratories for M. leprae viability testing around the world.

When large numbers of nu/nu mouse-derived M. leprae became available to the leprosy research community, radiorespirometry (RR), first described by Fransblau 1988 (5), was utilized to determine the viability of a bacterial preparation based on the rate of oxidation of $^{14}$C-palmitate by M. leprae. RR was found to correlate well with growth in MFP (27) and therefore was useful to evaluate the susceptibility of M. leprae to novel anti-leprosy drugs (6), a variety of environmental
conditions (27) as well as UV (26) and gamma radiation (1). This technique was also useful for characterization of the role of activated MΦ in host resistance to leprosy (19) and the effects of Schwann cells (M. leprae’s target in peripheral nerves) on M. leprae viability (7). However, the necessity to utilize large quantities of bacteria (10^7) for RR-based M. leprae viability assays limits the use of these assays for clinical purposes and in experiments where low numbers of bacteria are available (14, 19). In addition, RR requires the use of radioactive substances which is highly restricted in many areas of the world.

Recently, a fluorescence-based assay, LIVE/DEAD® BacLight™: Bacterial Viability assay for M. leprae viability determination was developed (14). This assay, based on cell membrane integrity, also correlated very well with MFP and RR assays for determination of M. leprae viability, however it also depends on the use of relatively large numbers of bacteria (10^6) for analysis and therefore limits it usefulness for clinical purposes and experimental conditions where only low numbers of bacteria are available.

PCR assays, based on amplification of various DNA sequences within genes encoding the 18 kDa, 36 kDa, 65 kDa, Ag 85 and the multicopy repeat sequence RLEP of M. leprae, have been successfully used to detect M. leprae in crude biological specimens even when low numbers of bacteria are present (11, 12, 15, 17, 28, 31). However, an important limitation of these DNA-based PCR assays is their inability to distinguish between viable and dead organisms and thereby provide information for drug efficacy in clinical settings and for short-term experimental procedures in vitro.
Detection of \textit{M. leprae} RNA has been proposed as a promising tool for rapid detection and measurement of the viability of pathogenic mycobacteria since degradation of RNA is relatively rapid upon cell death (3, 13). A previous study with \textit{M. tuberculosis} measured levels of \textit{M. tuberculosis} 85B (alpha antigen) mRNA, 16S rRNA, and IS6110 DNA using RT-PCR in patients’ sputa to ascertain whether they could serve as potential markers of response to chemotherapy (4). Results showed a rapid disappearance of \textit{M. tuberculosis} mRNA from sputum while DNA persisted in sputum from certain patients even after tuberculosis was cured. A preliminary study using the \textit{M. leprae} 16S rRNA as a predictor of viability showed this nucleic acid species to be a suitable target for detection of \textit{M. leprae} and its viability in clinical specimens using RT-PCR (8) including skin slits of treated patients (9, 16). Although these assays were able to detect \textit{M. leprae} viability in clinical specimens, they were not tested on paired samples from the same patient obtained pre- and during or post- MDT or in short-term experimental conditions where viability determination can be a critical denominator.

In the present study the utility of two RNA-based RT-PCR assays, one using the 16S rRNA as the RNA target and the other using a more labile \textit{sodA} mRNA as the target, was evaluated for both experimental and clinical usefulness. The \textit{sodA} mRNA transcript, encoding the superoxide dismutase A (ML0072c) of \textit{M. leprae}, was selected as the mRNA target for this assay because of the gene transcripts tested including: \textit{hsp18} (ML1795), \textit{gyrA} (ML0006), \textit{rpoB} (ML1891c), the \textit{sodA} transcript levels remained stable for at least 48 hr post harvesting from the mouse foot pad tissues but rapidly degraded after death of \textit{M. leprae} (data not shown). Also, since real-time RLEP-DNA based PCR had been previously been characterized as a rapid, objective, molecular
enumeration tool for detecting and quantifying bacterial numbers in an *M. leprae* preparation (29) it was chosen to serve as a normalizer for these assays.

Results from in vitro experiments demonstrated that both *sodA* mRNA/RLEP and 16S rRNA/RLEP RT-PCR assays were very good predictors of *M. leprae* viability in short-term experiments (up to 48 hr) when bacteria were exposed to lethal concentrations of rifampin (the only bacteriocidal drug in the MDT regiment for leprosy) in axenic medium or within infected mouse MΦ cultures. Assuming that the biological tests like RR and LIVE/DEAD® BacLight™ are gold standards for *M. leprae* viability, the 16S/RLEP appeared to be the most sensitive molecular assay for viability determination (*r* = 0.9817; *p* = 0.0005), even though *sodA*/RLEP assay also presented a significant correlation with gold standard methods (*r* = 0.9463; *p* = 0.043).

The ability of these assays to detect the effects of rifampin treatment further demonstrates their ability to differentiate between live and drug-killed *M. leprae* in axenic culture and within their host cells (MΦ). Therefore, these assays may be useful as rapid screening tools to identify effective antileprosy drugs as well as for experiments to study host parasite interactions. However, because of the inherent instability of the *sodA* mRNA species, resulting in rapid degradation following death of bacilli, the *sodA*/RLEP RT-PCR assay may be more useful for experiments which are designed to investigate early effects < 48 hr of drugs or immune factors on the viability of *M. leprae*. Preliminary results from our laboratory suggest that the *sodA*/RLEP assay can detect a loss in *M. leprae* viability as early as 12 hr post treatment (data not shown). These assays were not only found to be highly specific and but more sensitive than either RR (10^7) or LIVE/DEAD
BacLight® Bacterial Viability analysis ($10^6$) for determining the presence and viability of *M. leprae* under short-term experimental conditions.

However, the sodA/RLEP assay was only able to detect the presence of *M. leprae* in 25% of the pre-treatment biopsies tested. In contrast the 16S rRNA/RLEP assay was able to detect the presence of *M. leprae* in all pre-treatment biopsies analyzed. Several parameters may have contributed to these observed results. First, is the inherent labile nature of the mycobacterial mRNA compared to that of rRNA (22) which may be further affected by the and low degree of viability of *M. leprae* within the skin biopsies of leprosy patients in general (10). The second is the relative lower sensitivity detecting sodA versus 16S rRNA due to increased copy number of rRNA levels compared to that of mRNA.

In addition, results of the 16S rRNA/RLEP assay suggested a strong correlation between length of therapy and decline of *M. leprae* viability. Even though the case numbers were low, these results confirm the potential utility of this assay for monitoring anti-leprosy MDT therapy and thereby potentially identifying leprosy cases which are not responding to MDT due to drug resistance, non compliance or potential bacterial growth during or post treatment (relapse). In contrast, the lack of detectable *M. leprae* sodA gene transcripts in the majority of human biopsies demonstrated that this assay was not suitable for monitoring *M. leprae* viability in crude biological specimens.

In the present study, *M. leprae* viability was determined in skin biopsies based on 16S rRNA levels normalized by RLEP DNA levels. When RLEP data was used as an indicator of *M. leprae* numbers in an MB patient’s biopsy, there was a strong correlation between these results to that of
BI results obtained by microscopic examination of the skin slit smears from the same patients.

Thus, the RLEP PCR assay alone may be useful for defining the clinical form of the disease and potential up-or downgrading of disease status. However, no correlation was found between BI and 16S rRNA/RLEP levels, indicating that assessment of bacterial load per se does not reflect viability in most instances. This was anticipated because DNA-based PCR positive signals and the presence of acid-fast bacilli by microscopic examination persist in some cases years post-treatment but do not necessarily reflect the real impact of treatment on bacterial viability (25).

In conclusion, this study has identified additional tools for leprosy diagnosis and monitoring antileprosy drug efficacy for clinical purposes and for \textit{M. leprae} viability in short-term experimental studies which include the study of host cell/\textit{M. leprae} interactions. This has been made possible by the development of a procedure for the simultaneous isolation of \textit{M. leprae} RNA and DNA from the same sample and by incorporation of the previously described real-time RLEP PCR as a good predictor of \textit{M. leprae} numbers. Due to the stability and copy number of the 16S rRNA as well of the degradation of this molecule over time, the 16S rRNA/RLEP assay should be useful for determination of viable bacterial load in skin biopsies of MB leprosy patients and therefore may be important in determining MDT efficacy and the ability of patients to still be infectious post therapy. Hence, because of its high sensitivity and specificity this assay may also constitute a very sensitive and specific assay for early detection of \textit{M. leprae} in skin biopsies and therefore potentially a predictor of the clinical form of leprosy.
Acknowledgements

We wish to thank J.P. Pasqua for his excellent technical contributions to this work. This research was partially funded by the HRSA, BPHC, Division of the National Hansen’s Disease Programs, NIH/NIAID contract number Y1-AI-2646-01, CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).
References


Table 1. Primer and probe sequences for *M. leprae* ‘real time’ PCR and RT-PCR TaqMan assays.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16S Ribosomal RNA</td>
<td>ML16S rRNATaq-F</td>
<td>5’ GCA TGT CTT GTG GTG GAA AGC 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ML16S rRNATaq-R</td>
<td>5’ CAC CCC ACC AAC AAG CTG AT 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ML16S rRNATaq-Probe</td>
<td>5’ CAT CCT GCA CCG CA 3’</td>
</tr>
<tr>
<td>sodA mRNA</td>
<td>Superoxide Dismutase A</td>
<td>MLsodATaq-F</td>
<td>5’ ACC ACG CCG CAT ATG TCA 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLsodATaq-R</td>
<td>5’ CGC GTG CCT CGT CAA GT 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLsodATaq-Probe</td>
<td>5’ TGG CAA GCG CGT CAT TGA CAC CT 3’</td>
</tr>
<tr>
<td>RLEP</td>
<td>Repetitive Element</td>
<td>MLRLEPTaq-F</td>
<td>5’ GCA GCA GTA TCG TGT TAG TGA A 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLRLEPTaq-R</td>
<td>5’ CGC TAG AAG GTT GCC GTA T 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLRLEPTaq-Probe</td>
<td>5’ CGC CGA CGG CCG GAT CAT CGA 3’</td>
</tr>
</tbody>
</table>
Table 2. Comparison of various methods for *M. leprae* viability determination in infected murine MΦ.

<table>
<thead>
<tr>
<th><em>M. leprae</em>-infected MΦ Treatment</th>
<th>% <em>sodA</em>¹</th>
<th>% 16S rRNA²</th>
<th>% <em>BacLight</em>³</th>
<th>% Radiorespirometry⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin (20 µg/ml)</td>
<td>16 ± 4.1</td>
<td>57 ± 14.6</td>
<td>60 ± 5.5</td>
<td>49 ± 3.2</td>
</tr>
<tr>
<td>Ampicillin (50 µg/ml)</td>
<td>80 ± 5.5</td>
<td>90 ± 5.1</td>
<td>100 ± 6.6</td>
<td>97 ± 6.8</td>
</tr>
<tr>
<td>IFN-γ (100 IU)</td>
<td>1 ± 0.3</td>
<td>13 ± 0.6</td>
<td>23 ± 1.4</td>
<td>11 ± 1.3</td>
</tr>
</tbody>
</table>

1. % *sodA* = *sodA*/RLEP RT-PCR values of *M. leprae* in treated murine MΦ divided by that in untreated murine MΦ.

2. % 16S rRNA = 16S rRNA/RLEP RT-PCR values of *M. leprae* in treated murine MΦ divided by that in untreated murine MΦ.

3. % *BacLight* = LIVE/DEAD® *BacLight™* Bacterial Viability Assay values of *M. leprae* in treated murine MΦ divided by that in untreated murine MΦ.

4. % Radiorespirometry = day 7 cumulative counts per min (cpms) using Buddemeyer radiorespirometry of *M. leprae* viability in treated murine MΦ divided by that in untreated murine MΦ.
<table>
<thead>
<tr>
<th>Patient #</th>
<th>Biospy #</th>
<th>Leprosy Classification</th>
<th>MDT Treatment</th>
<th>BI</th>
<th>16S rRNA/RLEP (% ML viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>04-01</td>
<td>BL</td>
<td>Untreated</td>
<td>3.5</td>
<td>4314.3 ± 1395.7</td>
</tr>
<tr>
<td></td>
<td>05-01</td>
<td>1 yr</td>
<td></td>
<td>3.17</td>
<td>305.4 ± 112.5 (9)</td>
</tr>
<tr>
<td></td>
<td>06-01</td>
<td>2 yr</td>
<td></td>
<td>1.5</td>
<td>294 ± 76.4 (3)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>04-02</td>
<td>BL</td>
<td>Untreated</td>
<td>3</td>
<td>14.8 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>05-02</td>
<td>1 yr</td>
<td></td>
<td>2</td>
<td>8.8 ± 3.2 (59)</td>
</tr>
<tr>
<td></td>
<td>06-02</td>
<td>2 yr</td>
<td></td>
<td>2.8</td>
<td>8.3 ± 3.7 (56)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>03-03</td>
<td>LL</td>
<td>Untreated</td>
<td>1</td>
<td>50.2 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>04-03</td>
<td>1 yr</td>
<td></td>
<td>0.66</td>
<td>6.8 ± 4.9 (13)</td>
</tr>
<tr>
<td></td>
<td>05-03</td>
<td>2 yr</td>
<td></td>
<td>0.17</td>
<td>0.1 ± 0.05 (0.2)</td>
</tr>
<tr>
<td>Patient 4</td>
<td>00-04</td>
<td>LL</td>
<td>Untreated</td>
<td>1</td>
<td>337.7 ± 87.8</td>
</tr>
<tr>
<td></td>
<td>00-04</td>
<td>6 months</td>
<td></td>
<td>1</td>
<td>11.5 ± 4.0 (4)</td>
</tr>
<tr>
<td>Patient 5</td>
<td>03-05</td>
<td>LL</td>
<td>Untreated</td>
<td>1.2</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>04-05</td>
<td>1 year</td>
<td></td>
<td>0.7</td>
<td>1.3 ± 0.2 (28)</td>
</tr>
<tr>
<td>Patient 6</td>
<td>02-06</td>
<td>LL</td>
<td>Untreated</td>
<td>2.3</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>03-06</td>
<td>1 year</td>
<td></td>
<td>1.7</td>
<td>1.0 ± 0.1 (9.4)</td>
</tr>
<tr>
<td>Patient 7</td>
<td>03-07</td>
<td>LL</td>
<td>Untreated</td>
<td>2.3</td>
<td>39.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>04-07</td>
<td>1 year</td>
<td></td>
<td>1.7</td>
<td>0.6 ± 0.3 (1.6)</td>
</tr>
<tr>
<td>Patient 8</td>
<td>05-08</td>
<td>LL</td>
<td>Untreated</td>
<td>1.7</td>
<td>120.8 ± 23.2</td>
</tr>
<tr>
<td></td>
<td>06-08</td>
<td>1 year</td>
<td></td>
<td>1.2</td>
<td>0.5 ± 0.2 (0.4)</td>
</tr>
</tbody>
</table>

1 Leprosy patient classification according the Ripley-Jopling Scale (21).
2 Bacillary index (Acid fast bacilli count) of skin slits from leprosy patient (20)
Mean and standard deviation of 16S rRNA/RLEP RT-PCR values derived from cDNA of skin biopsies from untreated and treated leprosy patients and (% ML (M. leprae) viability) of each treated specimen assay derived by dividing the number of 16S rRNA/RLEP RT-PCR values of leprosy patients’ biopsies after treatment by that of untreated patient biopsy.
Figure Legends

Figure 1. Molecular viability analyses of rifampin-treated M. leprae. A) M. leprae sodA/RLEP levels; B) M. leprae 16S rRNA/RLEP levels. T-0 = untreated, T-48 hr = 48 h post rifampin treatment, T-1 wk = 1 wk post rifampin treatment, T-2 wk = 2 wk post rifampin treatment. The data are representative of three replicates for three independent experiments.

Figure 2. Comparison of M. leprae using real-time RLEP PCR and BI analyses in patients’ biopsies as function of pretreatment and post-treatment using Linear Pearson correlation between BI and DNA concentration from MB ($p = 0.001$; $r = 0.6942$).
Figure 2 Martinez et al.
ERRATUM

Molecular Determination of *Mycobacterium leprae* Viability by Use of Real-Time PCR

Alejandra N. Martinez, Ramanuj Lahiri, Tana L. Pittman, David Scollard, Richard Truman, Milton O. Moraes, and Diana L. Williams

*Laboratório de Hanseníase, Instituto Oswaldo Cruz—Fiocruz, Rio de Janeiro, Brazil, and HRSA, BPHC, Division of National Hansen’s Disease Programs, Laboratory Research Branch at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana*

Volume 47, no. 7, p. 2124–2130, 2009. Page 2125, column 1, line 4 of “Rifampin treatment in axenic medium” section: “(100 ng/ml)” should read “(8 mg/ml).”

Page 2125, column 1, line 6 of “Rifampin treatment in axenic medium” section: “20 μg/ml” should read “2 μg/ml.”

Page 2125, column 2, line 3: “20 μg/ml” should read “2 μg/ml.”

Page 2127, Table 2: The first entry in the first column should read “Rifampin (2 μg/ml).”

Page 2129: In the Acknowledgments section, the following sentence was inadvertently omitted: “We acknowledge the American Leprosy Mission for its support of a portion of this research.”