Original Article

Development and Evaluation of Real-Time RT-PCR Assay for Quantitative Estimation of Viable *Mycobacterium leprae* in Clinical Samples

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Abstract

Detection of live organisms by molecular methods has special significance in leprosy where causative organism can not be cultivated *in vitro.* Such techniques would be especially important for monitoring the progress of the disease. While real-time RT- PCR technology will be appropriate for this purpose, there is very little experience of use of such tools in leprosy. This study describes the development of a quantitative RT-PCR targeting 16S rRNA based on primers used in a semi quantitative RT-PCR and its application on clinical samples including slit scraping and biopsies. RNA was extracted from biopsies from 3 lepromatous leprosy (LL) cases and standard curve was generated by plotting crossing over point against the dilutions of input RNA quantity (number of bacilli used for RNA extraction). Real-time RT-PCR was performed for quantitative detection of live *M.leprae* in 28 slit (13/28 smear positive) scrappings and 32 biopsies (22/32 smear positive). Number of viable bacteria as estimated by solid stained bacilli and real-time PCR correlated (no difference $p>0.05$). The test achieved a theoretical analytical sensitivity limit of up to single live bacillus even considering 11.3% efficiency of RNA preparation which was calculated by spiking of known number of leprosy bacilli in non leprosy skin biopsies (PCR negative). All smear positive cases were positive by this assay. This assay appears to be a promising tool for detection and quantification of viable bacilli in selected clinical situations and should be of use even in smear negative cases also.

Key words : Quantitative estimation, Real-time RT-PCR, *M. leprae*, Clinical Sample

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Introduction

It has been more than a century when Armauer Hansen established that leprosy is caused by infection with an organism later named as *Mycobacterium leprae.* Treatment of leprosy is a great success story of modern times and most countries of world including India have achieved elimination as public health program (0.84 cases/10,000 in March 2006) (National Leprosy Eradication Programme 2007: http://nlep.nic.in/data.html) by using multi drug therapy (MDT). New case detection rate is however slow to decline (Visschedijk et al 2000), which may be due to long incubation period and also transmission of the disease may be continuing in some areas. Diagnosis of leprosy is usually based on clinical examination but it may be difficult to differentiate the leprosy from other diseases that cause similar symptoms, especially in areas where its incidence is low (Ooi et al 2001). Most common methods used for detection of bacillus in the laboratory is microscopic examination of slit skin smears which is, however, positive only in less than 20% of cases. Smear microscopy also does not allow accurate discrimination between viable v/s non viable and is unable to differentiate *M. leprae* from other mycobacteria that may be present in the lesions. As we are still not able to culture *M. leprae* in any acceptable *in vitro* medium, definite diagnosis and epidemiology of the disease is far from perfect. Conventional PCR, RT-PCR has been used with good success both in smear positive and negative cases (Kramme et al 2004). In case of DNA targeting PCR, signals have been shown to decline with therapy (Singh et al 1999). Thus better techniques are required for quantitative estimation and then accurately monitoring the effect of treatment. PCR specially targeting 16S rRNA (Cox et al 1991, Katoch et al 1992, Pattyn et al 1992) has been used as a tool for the diagnosis of leprosy. The 16S rRNA gene of *M. leprae* was selected as the target region for real-time RT-PCR because it had previously been shown to be appropriate for detection of live bacilli in clinical specimens (Sharma et al 1996). The performance of this real-time PCR shows its potential tool for quantitative estimation of viable bacilli. We report here the establishment of such assay, its technical and its preliminary clinical evaluation.

Materials and methods

Development of standard curve for quantitative RT-PCR : *M.leprae* specific primers (Jadhav et al 2005) targeting 16S rRNA were used for development of quantitative real-time RT-PCR and used for amplification of 16S rRNA from live *M.leprae.* Serial dilutions of homogenate (solid bacilli count by microscopy was 5.1E+4/ml homogenate) were prepared and RNA was extracted from three lepromatous leprosy cases (mean 5.1E+4 solid bacilli/ml) of homogenate were reverse transcribed and amplified by *M.leprae* specific primers targeting 16S rRNA gene by using LightCycler (Roche Version 3.5) and SYBR Green I reaction mix (Roche Diagnostics, Germany) with 200nM of each primer. Reverse transcription at 55°C for 10 min and initial de-naturation at 95°C for 30sec followed by 45 amplification cycles of annealing at 59°C and extension at 72°C with ramping of 20°C/Sec. Proportionally consistent increasing crossing over points (cycle number on witch intensity of dye become higher than the background) were observed while amplification of serial dilutions of RNA. Standard curve was generated by plotting the number of amplification cycles needed to obtain a detectable fluorescence signal (crossing over

point) against the serial dilutions of log10 solid bacillary count. The slope of the resulting linear relation was calculated by the LightCycler operation software (version 3.5) was then converted into the amplification efficiency using the formula: E=1 0–1/slope-1 (Rasmussen 2001).

Collection of clinical samples : To assess the clinical application of the real-time PCR, 60 [28 smear positive and 32 smear negative samples comprising of 32 skin biopsies (15/32 smear positive) and 28 slit (13/28 smear positive)] were collected from leprosy patients attending the OPD of Medical Unit-I of National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra and Model Rural Health Research Unit, Ghatampur, Kanpur, India. These were collected with informed consent of the patient for their involvement in the study. The specimens were collected in RNA Later (Ambion Inc., USA) solution under a sterilized condition preserved up to one week at 4°C. Patients were clinically diagnosed and classified according to Indian Association of Leprologists (IAL) classification (IAL 1982) by experienced clinicians of the Institute.

Processing and isolation of *M.leprae* total DNA and RNA : Biopsies were minced to a uniform consistency with sterile scissors and homogenized properly (homogenate should be able to pass through 26G needle) in Trizol solution (Invitrogen, USA) thoroughly in the pestle mortar. Slit samples suspended in RNA Later were centrifuged for 10 min at 1000xg and pellete was suspended in 1ml of Trizol reagent (Invitrogen, USA) and processed for RNA extraction as also followed for biopsy homogenates. 10µl of suspension was used for smear preparation. The smears were stained with Ziehl-Neelsen (ZN) stain and examined for acid-fast bacilli (AFB) and enumerated following standard method described by McRae and Shepard (1971). Total RNA including *M.leprae* RNA was extracted from skin biopsy homogenates as well as suspensions from slit scrapings with the protocol described using Trizol reagent (Invitrogen, USA) with slight modification at the precipitation step by increasing the time upto 2 hrs at -20°C. DNA was removed from these preparations by treating with 1µl DNase I using DNA-free ™ kit (Ambion Inc., USA) as specified by manufacturer and complete elimination of DNA was confirmed by no amplification in mock reaction. DNA free RNA aliquots were stored at -70°C.

Enumeration of live bacilli in clinical samples : Duplicate RT-PCR reaction was performed from 1µl RNA extracted from each samples by using same parameters used to generate standard curve. Target copy numbers in different type of samples were computed by relative quantification method by using standards of known copy number of 16S rRNA as external controls. At least one positive control of known copy number of template and negative control without template were run with every amplification reaction.

Results

Standard curve for estimation of live bacilli count : After real-time RT- PCR mean (n=3) crossing over points were plotted against mean log of estimated number of solid bacilli (Fig 1). Amplification efficiency on the basis of crossing over point was computed online at http://www.uri.edu/research/gsc/resources/cndna.html as described by Kramme et al (2004). Mean value of slope of the standard curve was -3.45 and efficiency was calculated to be 94.9% and theoretical minimum detection limit was observed to be up to single solid bacilli. This standard curve was

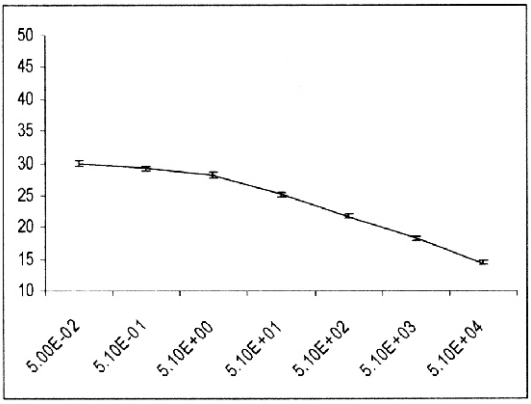

Fig. 1 : Standard curve of mean viable bacilli v/s crossing over point of real-time RT-PCR targeting 16S rRNA gene of *M.leprae*

used to calculate the live bacillary load in clinical samples including smear negative cases. This was correlated with viable count estimated from morphological index (counted on circular slide after ZN staining) of homogenate of smear positive cases.

To determine exactly the lower analytical sensitivity limit in clinical settings of our assay, known number (10 fold dilution of 5.1×10^4 bacilli) of bacilli were spiked to the tissue homogenate of healthy individual. RNA extraction followed by real-time RT-PCR assay was performed by using same protocol used for clinical samples and bacilli load was re-calculated. Mean efficiency of extraction was 11.33% which practically does not limit theoretical lower analytical sensitivity of the assay single *M. leprae* cell per clinical.

PCR sensitivity of the assay was 88.9% (24/28) in skin smear positive cases and 34.37% (11/32) in patients with skin smear negative results. Correlation coefficient was computed between live bacilli enumerated by direct microscopy count and with real-time RT-PCR. Mann-Whitney test was applied to see the difference between the performance of this assay and smear microscopy at 5% level of significance. No statistically significant difference ($P>0.05$) was observed in average live bacilli count by both techniques in biopsies as well as slit samples form smear positive LL/BL and biopsies from BB cases (Table 1).

Discussion

For conventional diagnosis and classification of leprosy smear microcopy has played a great role but it is difficult to estimate the number of viable bacilli and sensitivity of light microscopy is also very low. Molecular probes and amplification methods have been used in the last two decades for this purpose (Woods and Cole 1989; Katoch et al 1989, 1992, 1994; Pitulle et al 1990; Klatser et al 1991; Teske et al 1991; Cox

Table 1 : Positivity rates by direct detection using microscopy and real-time RT-PCR

		Direct microscopy		Real-time RT-PCR	
		No.	**%**	**No.**	**%**
Slit scrappings	+ve	13	46.43	19	67.86
(N=28)	-ve	15	53.57	9	32.14
		No.	**%**	**No.**	**%**
Biopsy	+ve	15	46.88	22	68.75
(N=32)	-ve	17	53.13	10	31.25

et al 1991; Misra et al 1995; Donoghoue et al 2001; Jadhav et al 2005; Phetsuksiri et al 2006). Among these methods RNA targeting molecular probe based methods has been reported higher sensitivity then DNA targeting methods (Sharma et al 1996). In this report, we describe a real-time RT-PCR assay for the detection and quantification of viable *M. leprae* in clinical specimens from leprosy cases. The test amplifies a portion of 16S rRNA of *M. leprae* which has been shown to be a sensitive and specific target region for PCR in many studies (Sharma et al 1996). Real-time PCR technology increases the speed and safety of PCR diagnostics by replacing agarose gel electrophoresis which otherwise consumes time, poses the risk of false positive results and enable us to quantitate the live bacilli load. A detailed evaluation of the analytical performance was done by spiking the tissue homogenate with known RNA copies. Assay was able to detect even single *M. leprae* cell per clinical sample with at least 95% efficiency considering the limitation factor of 11.3% for the efficiency of RNA preparation sample because of higher (2000-5000/live bacilli) copy number of 16S rRNA in a live bacilli. A linear relationship between the real-time PCR crossing point and the log10 input bacilli load was observed over a wide range of concentrations (5.1-5.1x 10^4/sample). Thus, this assay can be used to enumerate live *M. leprae* over the complete range (10^5 - single bacilli) of number of bacilli reported in infected animals (Job et al 1991, Rasmussen et al 2001) or humans tissue (Jamil et al 1993). Given the fact that no practicable and acceptable *in vitro* culture system for *M. leprae* is available, quantitation viable bacilli by real-time RT-PCR may thus become a useful tool in settings where the enumeration of bacteria in experimentally infected animals is required. Quantification of live bacilli has, moreover, been appeared to be useful for monitor the effect of therapy in patients. When our test was applied to clinical leprosy patients, similar range of positivity was observed as reported by others in MB and PB cases respectively e.g. 87.1% and 36.4% (Vander et al 1993), 89-99% and 4-74%, 92% and 61 % (Klatser et al 1991). Real-time detection dose not appear to increase the sensitivity of PCR despite its evidently high analytical sensitivity (Kramme et al 2004).

In areas with a high prevalence of leprosy, diagnosis will continue to rely on clinical and microscopical examination. PCR based assays have been reported to be helpful to improve the sensitivity and specificity of laboratory diagnosis in addition to smear microscopy (Williams et at 1992, Donoghoue et al 2001) and transmission of the disease by detection in the environment (Lavania et al 2008). In countries where leprosy is rare, confirmatory molecular assays may be useful to clarify unclear or suspected cases (Ooi and Moschella 2001). While signals of DNA based PCR decline after therapy (Wood and Cole 1989, Williams et al 1992), these may persist for a long period in a section of case (Singh et al 1999). As the number of clinical and research laboratories using real-time PCR instruments is increasing in our country, this test can easily be adapted to such laboratories. However, this assay needs to be evaluated by other users and correlation with other techniques based such as Mouse Foot Pad and ATP bioluminescence (Katoch et al 1989) needs to be studied.

No additional benefit for detection of bacilli or its component by this amplification method was apparent. This real-time PCR would, thus, be useful only for monitoring the treatment by assessment of viable bacillary load.

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