Journal of Advances and Scholarly Researches in Allied Education
Vol. 16, Issue No. 4, March-2019, ISSN 2230-7540

Assessment of the Viability of *Mycobacterium Leprae* from Soil Samples using Real Time RT-PCR Targeting 16S rRNA Gene

Dr. Vikram Singh[1]* Dr. Girija Kumari[2]

[1] Assistant Professor, Department of Medical Laboratory Technology, Amity Medical School, Amity University Haryana, Gurgaon, India

[2] Visiting Faculty, Department of Clinical Research, Amity Medical School, Amity University Haryana, Gurgaon, India

Abstract – Leprosy is an infectious and communicable disease caused by Mycobacterium leprae, which affects the skin and nerves. Leprosy has ceased to be a public health problem world-wide, after the successful implementation of effective chemotherapy and use of control measures. The present retrospective study was the part of MSc Biotechnology program and was conducted to assess the viability of Mycobacterium leprae from soil samples by Real time RT- PCR targeting 16S rRNA gene. In this retrospective study 40 soil specimens from 20 villages from Ghatampur region were taken and viability was assessed by Real time RT-PCR analysis targeting 16S rRNA gene region. RNA of M. leprae extracted and identified using specific M. leprae primers. PCR amplification was done and time period RT-PCR was accustomed observe viable M. leprae. Viable Mycobacterium leprae were detected by real time RT-PCR analysis targeting 16S rRNA gene region from 13out of 40 (32.5%) soil samples. The copy number of M.leprae estimated varied from 2.9x101 to 3.05x103 /100mg soil in the positive samples using the present protocol. Real time PCR targeting 16S rRNA appears to a promising approach for studying the presence of live M.leprae in the environment. This study therefore provides valuable data of presence of viable M. leprae in soil specimens, which might be of use in work the transmission dynamics in Hansen's disease.

Keywords: Leprosy, Mycobacterium Leprae, MDT, Real time RT-PCR, 16S rRNA gene

----------X----------

INTRODUCTION

Leprosy, additionally referred to as Hansen's malady, could be a chronic communicable disease caused by *Mycobacterium leprae*.

It is a rod-shaped slow-growing bacillus that is an obligate intracellular bacterium. The disease mainly affects the skin, the peripheral nerves, mucosal surfaces of the upper respiratory tract and the eyes (1). Leprosy is a fully treated disease with worldwide use of WHO recommended multi drug treatment (MDT) there is major decline in prevalence of disease. Multi drug treatment (MDT) with 3 antibiotics (dapsone, rifampicin, and clofazimine) treat multibacillary leprosy, while a modified MDT with two antibiotics (dapsone and rifampicin) is recommended for paucibacillary leprosy (2,3).

Currently 130 species of mycobacteria have been identified with more than 45 species of mycobacteria associated with human disease (4,5). As a result propagation of M.leprae has been restricted in animal models, including the armadillo and normal, athymic and gene knockout mouse. Growth of M.leprae on mouse footpads also provides a tool for accessing viability of preparation of bacteria and testing the drug susceptibility of clinical isolate. M.leprae stored at 33oC in 7H12 media has been shown to remain viable for weeks. Other than human the bacteria affects armadillos, mangable monkeys, Rabbies, mice ,Chimanzees and cynomologous Macaques. M.leprae was one of the first organism to be established as the cause of disease in humans (6).

The current prevalence of leprosy in India is 0.67/ 10, 000 population as on 31st March, 2018. This campaign is conducted once a year since 2016 – seventeen and covers around five hundredth of population of Republic of India. Health officers and activists celebrated thirteen years past once Republic of India declared that Hansen's disease had been eliminated as a public health concern. Alarm bells rang because the Central Hansen's disease Division of the health ministry reportable

that one hundred thirty five,485 new Hansen's disease cases were detected in Republic of India in 2017. That meant every four minutes somebody was diagnosed with leprosy in India (7). Recovery of M.leprae in both soil and water found to be higher in winter because mycobacteria get killed due to the odious cause of higher temp. and sterilizing effect of UV radiation during summer. Although there are not many reports of isolation of M. leprae from the environment, it has been known to survive for long period in the soil(8,9). The Presence of M. leprae DNA has also been reported in water samples in Indonesia in areas of high prevalence of the disease (10).

PCR analysis provides a sensitive and specific means to detect microorganisms in complex environmental samples such as soil (11,12) and water (13,14) in areas inhabited by leprosy patients in Brazil and India. The viability of M. leprae was assessed by its multiplication in footpads of wild type mice and showed that M. leprae can remain alive in wet soil for 46 days (15). Moreover, viability of M. leprae bacilli in soil from India has been studied by 16S ribosomal RNA gene analysis (16). Previous data showed that soil samples collected from patients' areas contained M. leprae 16S ribosomal RNA, suggesting the presence of viable M. leprae in the soil. In this retrospective study, we assess the viable Mycobacterium leprae from soil samples by Real time RT- PCR targeting 16S rRNA gene.

MATERIALS AND METHODS

The present retrospective study was the part of MSc Biotechnology program and was conducted to assess the viability of *Mycobacterium leprae* from soil samples by Real time RT- PCR targeting 16S rRNA gene. Total 40 samples from 20 different villages from Ghatampur, U.P, India were collected and processed in Laboratory.

Sample collection

Forty soil samples were collected from 20 different villages from around the houses of ''patient area'' (within 5 m) of Ghatampur area. Soil were dug from depth of 2 cm - 8 cm in areas without sun light and stored in 50 ml plastic containers with the help of khurpi and labeled bearing the village name. The person who collected the soil sample entered the soil collected in a register with the name of village. The collected samples were transferred to institute at room temperature within 2 days for the study and thereafter stored at 4 to 8 degree centigrade till farther processing.

Extraction of *Mycobacterium leprae* genomic RNA

Standard precautions were taken to forestall degradation of polymer by RNases throughout the purification steps including pretreatment of all glass ware with diethyl pyrocarbonate (DEPC) and baking them overnight at room temperature. All buffers were ready in DEPC-treated water before autoclaving. This was done to degrade any contaminating DNA.These samples were processed with slight modification in the protocol described by Miskin et al. 10ml of soil sample were taken in separate centrifuge tubes and Centrifuged at 10,000g for 30 min at 4˚C . Pellet was treated with 2ml extraction buffer (0.12 M sodium phosphate buffer + 10 mg lysozyme + 1% (w/v) mercaptoethanol; pH 8.0). This was agitated for 20 s. Homogenate was transferred to new vials containing 500 ml of 10% (w/v) SDS, vortexed and incubated at 80 C for 30 min with vigorous shaking every 10min.Repeat centrifugation was done at2800 _ g for15 min at 4 8C. Supernatant was emptied and pellet was re-removed with 2.5ml extraction cradle and again centrifuged. Supernatant was assembled and twofold volume of PEG 6000 (Merck, USA) was incorporated and kept for 2 h at room temperature for the precipitation of RNA.

Pellet was re-suspended in 1 ml DEPC-treated water. Hundred microlitres of 7.5 M potassium acetate was added to make a final concentration of 0.5 M. This was done for the precipitation of humic acids present in the sample (which interfere with the PCR) and centrifuged at 8000 g for 5 min. RNA was once more precipitated with double volume of chilled fermentation alcohol and unbroken at twenty 8C long. RNA was recovered by centrifugation at 8000 g for 15 min and dissolved in 20 ml of DEPC-treated water. RNA preparations were treated with DNase I (Ambion, USA) for removing any traces of contaminating DNA present in the soil samples. This was stored at 80 ˚C until use.

Real-time RT-PCR of 16S rRNA gene

The assay performed in 20 µl reaction volume. *M. leprae* ribonucleic acid in soil samples was additionally detected and quantified by amplification of 16S rRNA by time period reverse transcription PCR mistreatment ribonucleic acid amplification SYBR inexperienced I kit (Roche medical specialty, Germany), according to the standard protocols of the manufacturer. Briefly, master mix containing SYBR Green I reaction mix + RT-PCR enzyme mix + MgCl2 + 0.5 mM primers was prepared, and poured into PCR tubes. Two tubes were run for each sample 4 µl of RNA template was added to each tube. Total reaction volume (20µl) was transferred to Light Cycler capillaries, soft spinned and loaded to Light Cycler sample carousal. Positive management utilized in the experiment was inclusion polymer having *M.leprae* 16S rRNA sequence to visualize for positive amplification.

Amplifications of these samples were further quantified by real time RT-PCR using the

fluorescence dye SYBR Green I, targeting the same region of 16S rRNA.

SYBR Green I	-	4µl	
$MgCl_2$	-	2.4 µl (6mM)	
RT-PCR enzyme mix	-	0.4 µl	
Primer forward	-	0.2 µl	0.4 µM
Primer reverse	-	0.2 µl	0.4 µM
Nuclease free D/W	-	7.6 µl	
Template RNA		- 4 µl	

In real time PCR the concentration of primers kept low.

Primers and their sequences that we used are as follows-

Primer reverse- CCTGCACCGCAAAAAGCTTTCC ,

Primer forward- TCGAACCGAAAGGTCTCTAAAAAATC,

Primers (forward and reverse) specifically amplified 117 bp fragment of 16S r RNA of M.leprae.

Cycling profiles for 16s r RNA primer involved-

Reverse transcription at 55° C for 10 min and 95° C for 30 sec by PCR consisting of- Denaturation at 60° C for 15 sec ; Elongation at 72° C for 12 sec; Cooling at 40 C for 30 sec.

Relative Quantification-

Standard curve was plotted by crossing over point against the known copy of template (amplification efficiency 95.2%). The load of viable bacilli was calculated in the sample by importing the standard curve and relative quantification method. Standard curve was generated by plotting respective crossing over point against the log serial dilution (3.7×10^6) of log copy number of PCR product targeting 16 s r RNA genes. Relative quantification was performed to compute the mRNA copies specimen by using standard curve and sensitivity of the assay was let determined upto to 4 copies/sample.

RESULTS

Result of forty soil samples for detection of viable *M.leprae* by real time RT PCR targeting 16Sr RNA gene. Presented in Fig are summarized in Table-1 and 2. Only 13 (32.5%) out of 40 sample from 20 different villages were positive (copy number estimated to range from $2{,}9\times10^1$ to 3.05×10^3 /100mg soil).The result of 40 soil semples from 20 villages studied is presented in table 1& 2.

Table-1 Results of Real time RT PCR from soil samples

S. No.	Village Name	No. of Soil samples	Real Time RT- PCR,	
			Positive samples	% positivity Mean copy no.
1	Girsi	2	0	0 0
2	Kaitha	2	1	50% (1.78×10^3)
3	Sanuhi	2	1	50% (2.01×10^3)
4	Aagapur	2	1	50% (3.05×10^3)
5	Simaur	2	0	0 0
6	Nandana	2	2	100% (2.99×10^3)
7	Rdavli	2	1	50% 0
8	Sargown	2	2	100% (2.12×10^3)
9	Madha	2	0	0 0
10	Raypura	2	0	0 0
11	Newada Bharthua	2	0	0 0
12	Beerbal Ka Akabara	2	0	0 0
13	Mawai Bacchan	2	1	50% (1.01×10^3)
14	Machaila	2	0	0 0
15	Sukhapur	2	0	0 0
16	Shrinagar	2	1	50% (3.7×10^2)
17	Tilsada	2	0	0 0
18	Pahewa	2	1	50% (2.9×10^1)
19	Lakhankhera	2	0	0 0
20	Ittaura	2	2	100 % (2.66×10^2)
	Total	40	13	32.5% $(6.66\times10^3$

Table-2 Real Time Reverse transcription PCR targeting 16S rRNA gene of *M.leprae* of soil samples from different sources

Total number of samples	40
Positive RT PCR	13
Mean copy numbers	6.66×10^3

Development of standard curve for relative quantification: Serial dilutions of eluted PCR product (3.70E+6 to 3.7E+0 copies of 16S rRNA gene) were amplified and proportionally significant decreasing (slope = 3.47) was observed. The mean crossing over points and values were plotted. Mean crossing over points of 3 reactions of each dilution was plotted against log copy number to generate standard curve for relative quantification (Fig.1). Amplification efficiency of the assay was found to be 95.184% (Amplification= 1.952). It was observed that assay was thus able to amplify from single bacillus and this was positive with three rRNA templates.

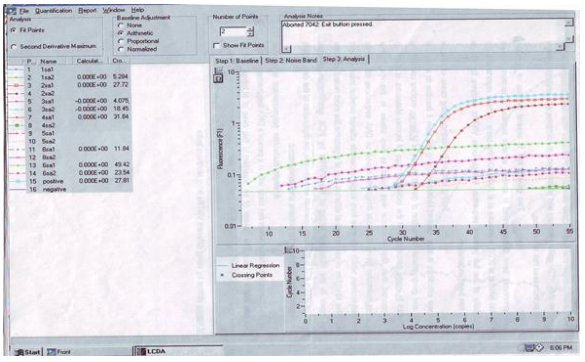

Fig 1. Real Time PCR amplification of 16S rRNA gene region from soil samples from Ghatampur field area

DISCUSSION

Soil bacterial populations are enormous and differing and are impacted by abiotic factors, for example, atmosphere and type, just as by neighborhood vegetation and other biotic information sources. In the same way as other different gatherings of microscopic organisms, some mycobacterium species are normal soil and water occupants. Soil Inorganic materials including cell free catalysts, overwhelming metals and follow components impact the microbes, fungi and other living inhabitants. Sickness has been considered to happen simply after introduction to a human case. In any case, proof has been aggregating that a natural non-human source may likewise be significant in transmission of the malady. Ebb and flow data substantiates the idea of condition wellspring of the ailment, and perceptions with monoclonal counter acting agent have demonstrated that phenolic glycolipid-I antigen, which is one of a kind to the M.leprae cell divider, is available in soil.

M. leprae may make due in the ecological from 2-46 days (8). A report from Izumi et al (17) utilizing PCR recommend a plausibility of quality of *M.leprae* DNA in water tests utilized by the infection patients. Such perceptions do demonstrate the capability of soil/water/condition to go about as a repository for M.leprae as beads and irresistible discharges from patient can taint these. Ongoing advancements in atomic science have made it conceivable to identify even little quantities of a particular microbes in a blended greenery in soil. Our investigation demonstrates the nearness of suitable *M.leprae* in soil. From 40 soil test 13 were seen to be continuous – RT PCR (2.5%).The mean duplicate number of bacilli recognized from each example was 6.66x103. A dirt corer was utilized to gather roughly 1.5 g of soil from 5 to 25 cm beneath the surface at each checked spot. The surface layer was not gathered in light of the fact that this layer is liable to quick condition change, including sanitization by the sun oriented radiation, as opposed to the subsurface soil.

It was seen that some dirt examples demonstrated the nearness of practical bacilli while different examples from a similar town did not contain any live bacilli, further trial should be performed to demonstrate the nearness of DNA of *M.leprae* from these examples and if present may demonstrate that *M.leprae* from contaminated individual are being shed into the earth which might utilize its feasibility with section of time.

The present examination in this manner demonstrated that by utilization of current sub-atomic apparatuses suitable *M.leprae* can be believed to get by in nature, anyway its particular job in the transmission of the sickness requires further investigations a very much characterized test populace and geological area.

CONCLUSION

Real time PCR targeting 16S rRNA appears to a promising approach for studying the presence of live *M.leprae* in the environment.The purpose of the study was to assess the viability of environmental mycobacteria and *M.leprae* from Ghatampur area by real time RT-PCR analysis targeting 16S rRNA.

In this study 40 soil specimens from 20 villages from Ghatampur region were taken and viability was assessed by Real time RT-PCR analysis targeting 16S rRNA gene region. (171-bp 16S rRNA gene fragment of *M.leprae* RNA) xs Viable *Mycobacterium leprae* were detected by real time RT-PCR analysis targeting 16S rRNA gene region from 13out of 40 (32.5%) soil samples. The copy number of *M.leprae* estimated varied from $2.9x10^1$ to $3.05x10^3$ /100mg soil in the positive samples using the present protocol.

ACKNOWLEDGEMENT

Authors are very thankful to concern authority (HOD & Supervisors) of Department of Microbiology & Molecular Biology, NJIL & OMD, ICMR, Agra, India for their kind support and relevant resources to complete this MSc Biotechnology dissertation work under their supervision and guidance.

REFERENCE

1. Bratschi, M. W., Steinmann, P., Wickenden, A. & Gillis, T. P. (2015). Current knowledge on Mycobacterium leprae transmission: a systematic literature review. Leprosy review 86, pp. 142–155.

2. WHO (1988). A guide to Leprosy Control. (www.who.int)

3. WHO India (2007). Leprosy situation in India epidemiological indicators. NLEP Indicators as on 31.06.07. http://www.whoindia.org /EN/Section3 /Section122_ 1461. Htm

4. Euzeby J.P. (2008). List of bacterial names with standing in nomenclature-Genus Mycobacterium. http://www.bacterio.cict.fr/m/mycobacteriu m.html.

5. Katoch VM, Lavania M, Chauhan DS et al (2007). Environmental mycobacteria: Friends and Foes. Environ Biol Conserv 12: pp. 87-100.

6. Araujo, S., Freitas, L. O., Goulart, L. R. & Goulart, I. M. (2016). Molecular evidence for the aerial route of infection of

Mycobacterium leprae and the role of asymptomatic carriers in the persistence of leprosy. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 63, pp. 1412–1420.

7. Bonnar, P. E. et. al. (2018). Leprosy in Nonimmigrant Canadian Man without Travel outside North America, 2014. Emerg Infect Dis 24, pp. 165–166.

8. Desikan K.V. (1977). Viability of Mycobacterium leprae outside the human body. Lepr Rev 48: pp. 231-235.

9. Desikan K.V. and Sreevasta (1995). Extended studies on the viability of Mycobacterium leprae outside the human body. Lepr Rev 66: pp. 287-295.

10. Matsuoka M., Izumi S., Budiawan T. et. al. (1999). Mycobacterium leprae DNA in daily using water as a possible source of leprosy infection. Indian J Lepr 71: pp. 61-67.

11. Turankar, R. P. et. al. (2014). Single nucleotide polymorphism-based molecular typing of M. leprae from multicase families of leprosy patients and their surroundings to understand the transmission of leprosy. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 20, pp. O142–149.

12. Turankar, R. P. et. al. (2016). Presence of viable Mycobacterium leprae in environmental specimens around houses of leprosy patients. Indian journal of medical microbiology 34, pp. 315–321.

13. Turankar, R. P. et. al. (2015). Comparative evaluation of PCR amplification of RLEP, 16S rRNA, rpoT and Sod A gene targets for detection of M. leprae DNA from clinical and environmental samples. International journal of mycobacteriology 4, pp. 54–59.

14. Holanda, M. V. et al. (2017). Presence of Mycobacterium leprae genotype 4 in environmental waters in Northeast Brazil. Revista da Sociedade Brasileira de Medicina Tropical 50, pp. 216–222.

15. Arraes, M. et. al. (2017). Natural environmental water sources in endemic regions of northeastern Brazil are potential reservoirs of viable Mycobacterium leprae. Memorias do Instituto Oswaldo Cruz 112, pp. 805–811.

16. Mohanty, P. S. et. al. (2016). Viability of Mycobacterium leprae in the environment and its role in leprosy dissemination. Indian journal of dermatology, venereology and leprology 82, pp. 23–27.

17. Izumi S. (1999). Detection of M.leprae in environment by PCR. Int J Epidimol 35: pp. 994-1000.

Corresponding Author

Dr. Vikram Singh*

Assistant Professor, Department of Medical Laboratory Technology, Amity Medical School, Amity University Haryana, Gurgaon, India

vsmicroaiims@gmail.com