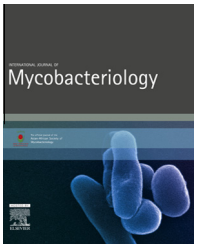
International Journal of Mycobacteriology 4 (2015) 54–59

HOSTED BY

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/IJMYCO



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

Ravindra P. Turankar [1], *Shradha Pandey* [1], *Mallika Lavania*, *Itu Singh*, *Astha Nigam*, *Joydeepa Darlong*, *Fam Darlong*, *Utpal Sengupta* [*]

Stanley Browne Laboratory, TLM Community Hospital, Nand Nagari, Delhi 110093, India

ARTICLE INFO

Article history:
Received 30 October 2014
Accepted 2 November 2014
Available online 23 January 2015

Keywords:
M. leprae
Comparison of PCR positivity
Diagnostic of leprosy
Leprosy and different gene target
Clinical samples
Environmental samples

ABSTRACT

Purpose: PCR assay is a highly sensitive, specific and reliable diagnostic tool for the identification of pathogens in many infectious diseases. Genome sequencing *Mycobacterium leprae* revealed several gene targets that could be used for the detection of DNA from clinical and environmental samples. The PCR sensitivity of particular gene targets for specific clinical and environmental isolates has not yet been established. The present study was conducted to compare the sensitivity of *RLEP*, *rpoT*, *Sod A* and *16S rRNA* gene targets in the detection of *M. leprae* in slit skin smear (SSS), blood, soil samples of leprosy patients and their surroundings.

Method: Leprosy patients were classified into Paucibacillary (PB) and Multibacillary (MB) types. Ziehl–Neelsen (ZN) staining method for all the SSS samples and Bacteriological Index (BI) was calculated for all patients. Standard laboratory protocol was used for DNA extraction from SSS, blood and soil samples. PCR technique was performed for the detection of *M. leprae* DNA from all the above-mentioned samples.

Results: *RLEP* gene target was able to detect the presence of *M. leprae* in 83% of SSS, 100% of blood samples and in 36% of soil samples and was noted to be the best out of all other gene targets (*rpoT*, *Sod A* and *16S rRNA*). It was noted that the *RLEP* gene target was able to detect the highest number (53%) of BI-negative leprosy patients amongst all the gene targets used in this study.

Conclusion: Amongst all the gene targets used in this study, PCR positivity using *RLEP* gene target was the highest in all the clinical and environmental samples. Further, the *RLEP* gene target was able to detect 53% of blood samples as positive in BI-negative leprosy cases indicating its future standardization and use for diagnostic purposes.

[*] Corresponding author. Tel.: +91 9212761651, +91 11 22594295.
E-mail address: usengupta2002@yahoo.com (U. Sengupta).
[1] Equally contributed.

Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2014.11.062

Introduction

Leprosy is a chronic infectious disease of humans caused by *Mycobacterium leprae* and was discovered by G.A. Hansen in 1873. *M. leprae* is a non-cultivable mycobacteria, and diagnosis of the disease is based on its clinical, histopathological characteristics and finding the bacteria in skin scrapings and in biopsies taken from the patients. Due to its long incubation period and because very early lesions often do not satisfy the cardinal signs of leprosy, it becomes difficult to diagnose the disease in the early stages. Therefore, there is an urgent need to develop a tool for diagnosing the disease early so that the patient can be covered with chemotherapy under the control program. The diagnosis of leprosy is routinely based on clinical symptoms and finding acid-fast bacilli (AFB) in smears of skin scrapings. AFB staining technique requires the presence of at least 10^4 organisms per gram of tissue for its reliable detection under the microscope [1] and thus the organisms have a very low sensitivity of detection, especially in patients with intermediate lesions and at the Tuberculoid (TT)/Borderline Tuberculoid (BT) end of the disease spectrum where AFBs are rare or absent.

Several attempts have been made in the past to establish a test for the diagnosis of early leprosy; however, none of the tests were successful in diagnosing more than 60% of early cases of leprosy [2–5] and, therefore, til today, there is no laboratory-based specific and sensitive assay for the detection of early leprosy.

Modern molecular methods like amplification by polymerase chain reaction (PCR) are more specific and sensitive for detecting bacillary DNA in clinical samples [6]. During the last 30 years, PCR methods have been developed to amplify different gene targets of *M. leprae*, but it is not known which target is most suitable for the diagnosis of leprosy. *RLEP* was found to be very sensitive and specific for *M. leprae* and detects 10 fg of purified *M. leprae* DNA [7,8]. *16S rRNA* is a gene conserved in bacteria which codes for the 16S part of the ribosome [9,10]. It has been used for RT-PCR targeting *16S rRNA* for identification of viable bacilli from patients. Martinez et al. reported that with regard to *16S rRNA* and *RLEP*, RT-PCR acts as a better target compared with *superoxide dismutase* (*Sod A*) and *RLEP* RT-PCR for viability testing of *M. leprae* [11].

Enormous numbers of leprosy bacilli are expelled into the environment from the nasal discharges of lepromatous patients [12]. There is also evidence to support the excretion of bacilli from skin lesions [13,14]. A single mouth-wash of an LL patient may discharge 1.6 million *M. leprae* [15]. The risk of transmission of leprosy in the community, therefore, is subject to the availability of leprosy cases and other related environmental factors. There are evidences to support that *M. leprae* is able to survive for many days in the environment. Soil is known to be a medium which can preserve a variety of microorganisms [16]. Although PCR has already been applied for the detection of *M. leprae* for many years, it has been used mainly in samples from biopsies and SSS of leprosy and suspected cases and not in environmental samples.

The aim of the present study is to evaluate the usefulness of the PCR method on different clinical and environmental samples using different gene targets, such as *RLEP*, *rpoT*, *sod A* and *16S rRNA* genes, and compare their efficiency in PCR positivity in clinical samples (blood and SSS) and environmental samples (soil) which were obtained from patients and from their surroundings.

Materials and methods

Ethical approval

Informed consent was obtained from all the patients, and the study was approved by the Ethical Committee of The Leprosy Mission Trust India.

Recruitment of patients

Thirty newly diagnosed AFB negative PB and 30 MB leprosy cases as per standard criteria of the World Health Organisation (WHO) were enrolled from the Outpatient Department of the TLM Hospital Shahdara, Delhi and Purulia, West Bengal. Grading of the bacterial load was determined by ZN staining of SSS. Fifty soil samples were collected in sterile plastic containers following a procedure described earlier [17] from residing areas and/or around the houses of leprosy patients staying in different villages of different blocks of the Purulia district of West Bengal (Table 1).

Sampling area

Different blocks of Purulia, West Bengal and Shahdara of Delhi were selected for sampling.

Slit skin smear sample collection

SSS samples were collected after taking four horizontal scrapes of tissue from an incision (5 mm long and 2 mm deep) made with the help of a sterile surgical scalpel blade (No. 15) on the left and right earlobes and skin lesions without any contamination with blood along the skin-slit part. The tissue material thus obtained on the blade by scraping was placed in 700 μl of 70% ethanol and mixed well in a micro-centrifuge tube. Later the SSS suspensions were kept at 4 °C for further use.

Collection of blood samples

Blood samples (2 ml each) were withdrawn from patients by antecubital venipuncture and collected in an EDTA vial;

Table 1 – SSS samples of PB and MB leprosy patients.

BI grading	Number of PB cases	Number of MB cases	Total number of cases
0	30	–	30
1+	–	6	6
2+	–	6	6
3+	–	6	6
4+	–	6	6
5+	–	6	6
Total	30	30	60

BI = Bacteriological Index; PB = Paucibacillary; MB = Multibacillary.

Table 2 – Blood samples of PB and MB leprosy patients.

BI grading	Number of PB cases	Number of MB cases	Total number of cases
0	30	–	30
1+	–	6	6
2+	–	6	6
3+	–	6	6
4+	–	6	6
5+	–	6	6
Total	30	30	60

BI = Bacteriological Index; PB = Paucibacillary; MB = Multibacillary.

300 μl of the blood sample was used for DNA extraction by using standard protocol as mentioned below (see Table. 2).

Collection of soil sample

50 soil samples were collected in clean plastic containers (10 g each) with the help of a hand trowel and labelled with a site code and the village name. Specific locations for the collection of soil samples were the bathing place, common sitting place, near the entrance of the house, areas around the house used for washing and a place near the bore well. Collected samples were transported to the laboratory at room temperature (within 2 days) and stored at 4–8 °C until further processing.

Extraction of DNA from slit sample

M. leprae DNA was isolated from SSS samples by Proteinase K lysis method [18]. Briefly, SSS samples were collected and kept in 70% ethanol. Then all the tubes were again centrifuged at 10,000× rpm for 10 min, and the supernatant was discarded. The pellet was dried in an incubator at 37 °C for 2 h; 200 μl of lysis buffer was added to the dried pellet. The pellets with lysis buffer were kept at 60 °C for overnight or 16 h in a shaking water bath for lysis. The lysis reaction was terminated by inactivating Proteinase K by incubation at 97 °C for 15 min. Lysates were stored at −20 °C until further use.

Extraction of DNA from blood sample

DNA was extracted and purified using a Master Pure DNA extraction Kit (Epicentre Technologies, USA) according to the manufacturer's instructions.

Extraction of DNA from soil sample by using kit

DNA was extracted and purified using a Power Soil DNA Isolation Kit (MO BIO-laboratories, USA) according to the manufacturer's instructions.

Polymerase chain reaction

DNA PCR amplification was carried out by using different gene targets of *M. leprae*. A total of 25 μl of reaction volume that contained 2 μl of template DNA, primers at a final concentration of 0.5 μM (forward and reverse) and 1× Genei Mix (Merck India). *M. leprae*-specific *RLEP* primers sequences PS1 5′-TGC ATG TCA TGG CCT TGA GG-3′ and PS 2 5′-CAC CGA TAC CAG CGG CAG AA-3′ were used [7]. Amplification consisted of first stage of single cycle of denaturation at 95 °C for 5 min followed by second stage of 35–45 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, and final stage of single cycle of 10 min at 72 °C. The *M. leprae* specific *16S rRNA* primers (P2: 5′-TCG AAC GGA AAG GTC TCT AAA AAA TC-3′ and P3: 5′-CCT GCA CCG CAA AAA GCT TTC C-3′) described earlier were used [19]. PCR reaction without DNA was used as the negative control (reagent control). Purified *M. leprae* DNA was used as the positive control. The amplification was carried out in a thermal cycler (Corbett Research, Australia) under the following conditions of 95 °C for 5 min for initial denaturation followed by 37 cycles, each cycle consisting of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 2 min and extension at 72 °C for 3 min followed by a final extension at 72 °C for 10 min. *rpoT* PCR amplification was carried out by *using M. leprae* DNA as per the protocol described earlier [20]. *rpoT* Primer A (VIC-5′-ATG CCGAACCGGACCTC-GACGTTGA-3′) and B (5′-TCGTCTTCGAGGTCGTCGAGA-3′) (Gene Bank Accession No. AB019194) which were used for amplification span the 91 bp (containing 3 repeats) or 97 bp (containing 4 repeats) fragments of the *rpoT* gene target. The amplification was carried out in a thermal cycler (Corbett Research, Australia) under the conditions of 95 °C for 5 min for initial denaturation followed by 40 cycles, each cycle consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 2 min and extension at 72 °C for 3 min followed by a final extension at 72 °C for 10 min. The *M. leprae* specific *Sod A* primers (Sod A-F GGCCAGGTTCTTCTCGTTCA and *Sod A*-R CGCCGCATATGTCAAAGGTG) described earlier were used [21]. The amplification was carried out in a thermal cycler (Corbett Research, Australia) under the following conditions of 95 °C for 5 min for initial denaturation followed by 37 cycles, each cycle consisting of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 90 s and extension at 72 °C for 60 s followed by a final extension at 72 °C for 10 min. PCR product containing an amplified fragment of the target region was electrophoresed in agarose (Sigma) gel using Tris–Borate–EDTA buffer at 100 V constant voltage.

Results

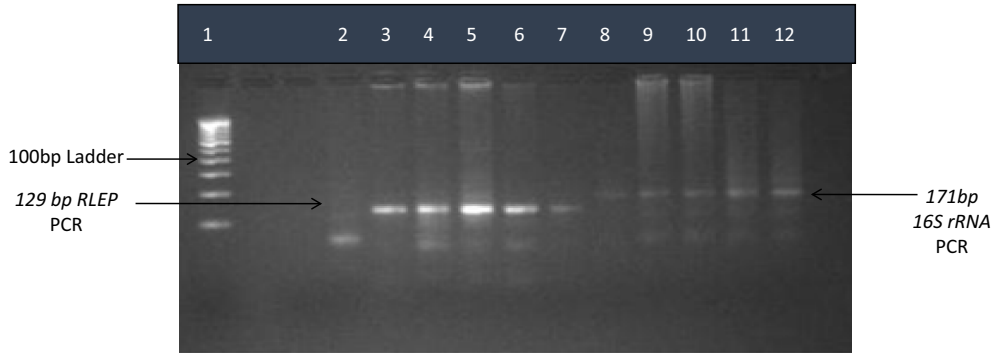
It is noted from Table 3 that *RLEP* showed the highest (83%) positivity for *M. leprae* in SSS compared with *rpoT* (70%), *Sod A* (57%) and *16S rRNA* (60%) gene targets, respectively (Figs. 1 and 2). When PCR positivity for *M. leprae* in blood samples was compared, it was observed that all the BI positive cases were *RLEP* positive (100%), while the positivity for other targets were 80%, 90%, 43% for *rpoT*, *Sod A* and *16S rRNA*, respectively (Table 4).

It was interesting to note that *RLEP* showed the highest positivity of 30% of SSS and 53% of blood samples of BI negative PB cases amongst all the gene targets (Table 5).

All 50 soil samples were screened by using the aforementioned *M. leprae* gene targets. It was observed that PCR positivity of *M. leprae* DNA from soil samples was the highest using the *RLEP* region (36%) as compared with *16S rRNA* gene (30%), *Sod A* (0%), and *rpoT* (0%) (Fig. 3).

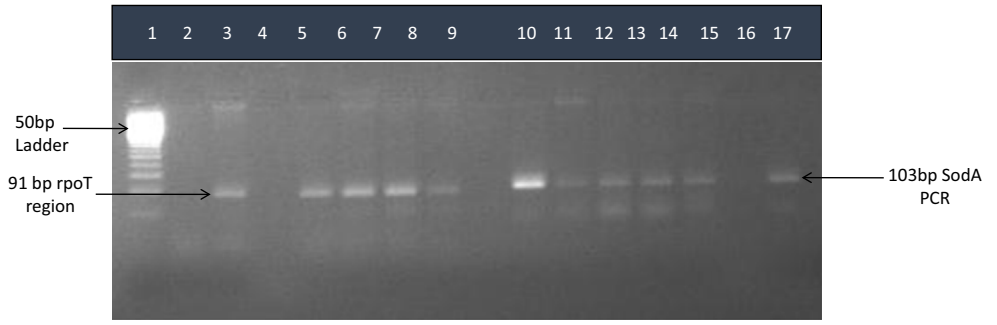
Table 3 – PCR positivity from SSS samples.

BI	Total number	*RLEP* PCR positivity	*rpoT* PCR positivity	*Sod A* PCR positivity	*16S rRNA* PCR positivity
1+	6	4 (67%)	4 (67%)	2 (33%)	2 (33%)
2+	6	4 (67%)	4 (67%)	3 (50%)	2 (33%)
3+	6	5 (83%)	4 (67%)	3 (50%)	4 (67%)
4+	6	6 (100%)	4 (67%)	4 (67%)	3 (50%)
5+	6	6 (100%)	5 (83%)	5 (83%)	5 (83%)
Total	30	25 (83%)	21 (70%)	17 (57%)	18 (60%)



PCR products were electrophoresis on 2% Agarose gel. Samples were Lane 1: 100bp ladder, Lane 2: Negative control; Lane 3: positive control, Lane 4-7: RLEP PCR product using SSS samples, Lane 8-12: *16S rRNA* PCR product using SSS samples.

Fig. 1 – Shows PCR amplification of representative *M. leprae* gene targets of RLEP, 16S rRNA gene targets from SSS samples.



PCR products were electrophoresis on 3% Agarose gel. Samples were Lane 1: 50bp ladder, Lane 2: Negative control, Lane 3: positive control, Lane 4-9: SSS samples for rpoT region, Lane 10-15: SSS samples for Sod A region, Lane 16: negative control, Lane 14: positive control.

Fig. 2 – Shows PCR amplification of representative *M. leprae* gene targets of *rpoT*, *Sod A* gene targets from SSS samples.

Discussion

Although leprosy is one of the oldest infectious diseases that has inflicted man from biblical times, many questions on modes of entry, incubation period of the disease and early manifestation of the disease are still not known. Over the last few years, several articles have been published on PCR-mediated amplification of *M. leprae* DNA, and these data suggest that PCR could be a useful tool for the detection of infection with this pathogen. Identification of *M. leprae* is difficult from a biological sample due to its inability of the leprosy bacillus to grow *in vitro*. The diagnosis of leprosy is based on microscopic detection of AFB in tissue smears, in combination with histopathological and clinical evaluation. Because acid-fast staining requires at least the presence of 10^4 organisms per gram of tissue for reliable detection [22], sensitivity of detection is low, particularly for the majority of PB patients wherein AFB are rare or absent.

In the past 30 years, definitive identification of *M. leprae* has been possible through the development of methods for the amplification and identification of *M. leprae* DNA in clinical and environmental samples using PCR. The techniques have been applied not only to skin biopsy samples, but also to several different types of specimens, such as skin smears,

Table 4 – PCR positivity from blood samples by using different gene targets of *M. leprae*.

BI	Total Number	*RLEP* PCR positivity	*rpoT* PCR positivity	*Sod A* PCR positivity	*16S rRNA* PCR positivity
1+	6	4 (67%)	4 (67%)	2 (33%)	2 (33%)
2+	6	4 (67%)	4 (67%)	3 (50%)	2 (33%)
3+	6	5 (83%)	4 (67%)	3 (50%)	4 (67%)
4+	6	6 (100%)	4 (67%)	4 (67%)	3 (50%)
5+	6	6 (100%)	5 (83%)	5 (83%)	5 (83%)
Total	30	25 (83%)	21 (70%)	17 (57%)	18 (60%)

Table 5 – Comparison of BI negative SSS and blood samples with PCR assay by using different gene targets.

Samples	BI	Total number	*RLEP* PCR positivity	*16S rRNA* gene positivity	*SodA* gene positivity	*rpoT* positivity
SSS samples	Neg	30	9 (30%)	5 (17%)	2 (7%)	3 (10%)
Blood samples	Neg	30	16 (53%)	3 (10%)	6 (20%)	0 (00%)

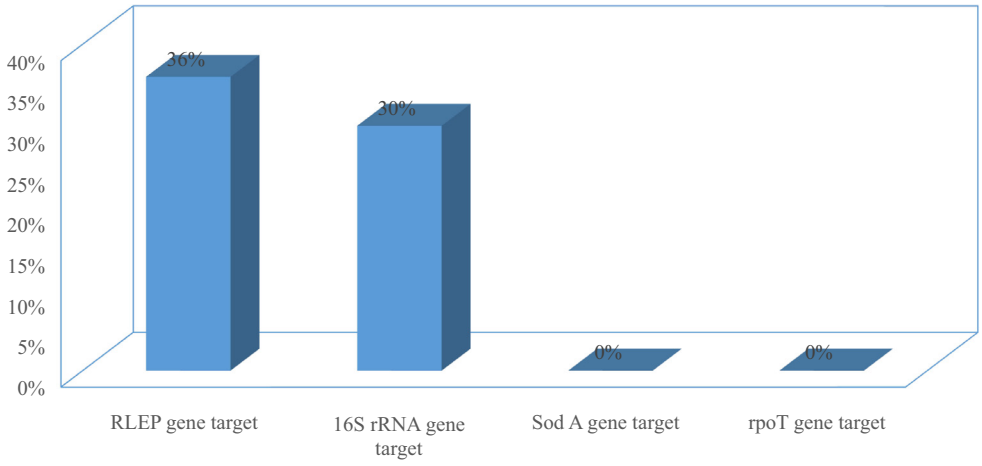


Fig. 3 – PCR positivity of *M. leprae* DNA from soil samples by using different gene targets.

nerves, urine, mouth washing, nasal swabs, blood, and ocular lesions [23–30]. Different sequences were used as targets for PCR, such as genes encoding antigen 85 [26], *16S rDNA* [21], *RLEP* [8] and *sod A* [21]. The *M. leprae* chromosome contains a family of dispersed repeats (*RLEP*) of variable structure and unknown function [31], and it was noted that the presence of the sequence of *RLEP* in *M. leprae* chromosome was repeated 28 times [21]. *RLEP* RT-PCR acts as a better target compared with *Sod A* for viability testing of *M. leprae*. It has demonstrated that the level of the *Sod A* gene transcript was reduced to only 5% compared with *16S rRNA* gene, which was reduced to 38% within 48 h after rifampicin treatment. However, after a week of treatment, both were essentially at the background levels. *rpoT* gene, a homolog of the mycobacterial principle sigma factor, has been found recently to be present in three copies in most strains of *M. leprae*, including Tamil Nadu strain (TN), but in four copies in others [20,32].

There are very few studies that focus on the detection of *M. leprae* from whole-blood samples. In the present study, the efficacy of PCR of several *M. leprae* gene targets in blood and SSS samples of AFB-negative PB and AFB-positive MB leprosy patients and soil samples collected from the surroundings of patients were investigated. *RLEP* PCR positivity from both blood and SSS samples was found to be the highest (100%, 83%) when compared with other gene targets of *M. leprae*. These results suggest that *RLEP* PCR could be used for the early detection of leprosy cases and for follow-up of the patients to assess bacterial load reduction during chemotherapy. Even though 83% of *RLEP* PCR positivity was observed from SSS samples of MB cases, all these cases were found to be positive in PCR from blood samples. Further, while in this study *RLEP* PCR was 100% positive, it was shown to be 53% positive in MB cases of leprosy [24]. In addition, the present study could also diagnose 53% of BI negative PB cases.

PCR positivity was highest among all the clinical and environmental samples by using the *RLEP* region of *M. leprae*. All these findings for the first time clearly demonstrated that the *RLEP* target is the most suitable out of all the other targets for identifying *M. leprae* gene either in the clinical samples or in the environmental samples. Future research may be directed using *RLEP* PCR in developing and standardizing an early diagnostic assay for leprosy.

Declaration of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We acknowledge the infrastructural and financial support rendered by The Leprosy Mission Trust India (TLMTI) to carry out this research work at Stanley Browne Research Laboratory – The Leprosy Mission Community Hospital, Shahdara – New Delhi. We also acknowledge Dr. Sunil Anand, Director – TLMTI, Dr. Annamma John, Research Head Coordinator, TLMTI for their support and encouragement. We also wish to acknowledge support of the Superintendent and staff of TLM Hospital, Purulia. We would also like to extend our thanks to Mr. Atul Roy for assistance in sample collection.

REFERENCES

[1] C.C. Shepard, D.H. McRae, A method for counting acid-fast bacteria, Int. J. Lepr. 36 (1968) 78–82.
[2] U. Sengupta, *Mycobacterium leprae* antigens and their utility in immunodiagnostics, Trop. Med. Parasitol. 41 (1990) 361–362.
[3] O. Parkash, V. Chaturvedi, B.K. Girdhar, U. Sengupta, A study on performance of two serological assays for diagnosis of leprosy patients, Lepr. Rev. 66 (1995) 26–30.
[4] S. Buhrer-Sekula, H.L. Smits, G.C. Gussenhoven, J. van Leeuwen, S. Amador, T. Fujiwara, et al, Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk developing leprosy, J. Clin. Microbiol. 41 (2003) 1991–1995.
[5] M.S. Duthie, W. Goto, G.C. Ireton, S.T. Reece, L.P.V. Cardoso, C.M.T. Martelli, et al, Use of protein antigens for early serological diagnosis of leprosy, Clin.Vaccine Immunol. 14 (2007) 1400–1408.
[6] M.Y.L. De Wit, W.R. Faber, S.R. Krieg, J.T. Douglas, S.B. Lucas, N. Montreewasuwat, et al, Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues, J. Clin. Microbiol. 29 (1991) 906–910.
[7] H.D. Donoghue, J. Halto, M. Spigelman, PCR primer that can detect low levels of *mycobacterium leprae* DNA, J. Med. Microbial. 50 (2001) 177–182.
[8] R.W. Truman, P.K. Andrews, N.Y. Robbins, L.B. Adams, J.L. Krahenbuhl, T.P. Gillis, Enumeration of *Mycobacterium leprae* using real-time PCR, PLoS Negl. Trop. Dis. 2 (2008) 328.
[9] M. Kurabachew, A. Wondimu, J.J. Ryon, Reverse transcription PCR detection of *M. leprae* in clinical specimens, J. Clin. Microbiol. 36 (1998) 1352–1356.
[10] S.R. Pattyn, D. Uris, M. Iven, R.J.W. Rees, P. James, Polymerase chain reaction amplifying DNA coding for species specific rRNA of *Mycobacterium leprae*, Int. J. Lepr. 60 (1992) 234–243.
[11] A.N. Martinez, C.F. Britto, J.A. Nery, Molecular determination of *Mycobacterium leprae* viability by use of real-time PCR, J. Clin. Microbiol. 47 (2009) 2124–2130.
[12] T.F. Davey, R.J. Rees, The nasal discharge in leprosy: clinical and bacteriological aspects, Lepr. Rev. 45 (1974) 121–134.
[13] C.K. Job, J. Jayakumar, M. Kearney, T.P. Gillis, Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR, Am. J. Trop. Med. Hyg. 78 (2008) 518–521.
[14] G. Kotteswaran, C.J. Chacko, C.K. Job, Skin adnexa in leprosy their role in the dissemination of *M. leprae*, Lepr. India 52 (1980) 475–481.
[15] G. Ramu, Central JALMA Institute for Leprosy, Annual Report, Lepr. India 53 (1981) 307.
[16] K.V. Desikan, Sreevatsa, Extended studies on the viability of *Mycobacterium leprae* outside the human body, Lepr. Rev. 66 (1995) 287–295.
[17] R.P. Turankar, M. Lavania, M. Singh, et al, Dynamics of *Mycobacterium leprae* transmission in environmental context: deciphering the role of environment as a potential reservoir, Infect. Genet. Evol. 12 (2012) 121–126.
[18] R.S. Jadhav, M. Macdonald, G. Bjune, et al, MILEP2 study group, simplified PCR detection method for nasal *Mycobacterium leprae*, Int. J. Lepr. Other Mycobact. Dis. 69 (2001) 299–307.
[19] R.S. Jadhav, R.R. Kamble, V.S. Shinde, S. Edward, V.K. Edward, Use of reverse transcription polymerase chain reaction for the detection of *Mycobacterium leprae* in the slit-skin smears of leprosy patients, Indian J. Lepr. 77 (2005) 116–127.
[20] M. Matsuoka, S. Maeda, M. Kai, N. Nakata, G.T. Chae, T.P. Gillis, K. Kobayashi, et al, *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes, Int. J. Lepr. Other Mycobact. Dis. 68 (2000) 121–128.
[21] A.N. Martinez, R. Lahiri, T.L. Pittman, D. Scollard, R. Truman, et al, Molecular determination of *Mycobacterium leprae* viability by use of real-time PCR, J. Clin. Microbiol. 47 (2009) 2124–2130.
[22] C.C. Shepard, D.H.A. McRae, A method for counting acid-fast bacteria, Int. J. Lepr. 36 (1968) 78–82.
[23] M.R. Jardim, S.L. Antunes, A.R. Santos, O.J. Nascimento, J.A. Nery, et al, Criteria for diagnosis of pure neural leprosy, J. Neurol. 250 (2003) 806–809.
[24] A.R. Santos, A.B. De Miranda, E.N. Sarno, P.N. Suffys, W.M. Degrave, Use of PCR-mediated amplification of *Mycobacterium leprae* DNA in different types of clinical samples for the diagnosis of leprosy, J. Med. Microbiol. 39 (1993) 298–304.
[25] E.C. Almeida, A.N. Martinez, V.C. Maniero, A.M. Sales, N.C. Duppre, et al, Detection of *Mycobacterium leprae* DNA by polymerase chain reaction in the blood and nasal secretion of Brazilian household contacts, Mem. Inst. Oswaldo. Cruz 99 (2004) 509–511.
[26] A.N. Martinez, C.F. Britto, J.A. Nery, Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy, J. Clin. Microbiol. 44 (2006) 3154–3159.
[27] F.A. Shamsi, I.A. Chaudhry, M.O. Moraes, A.N. Martinez, F.C. Riley, Detection of *Mycobacterium leprae* in ocular tissues by histopathology and real time polymerase chain reaction, Ophthalmic Res. 39 (2007) 63–68.
[28] M.Y. de Wit, J.T. Douglas, J. McFadden, P.R. Klatser, Polymerase chain reaction for detection of *Mycobacterium leprae* in nasal swab specimens, J. Clin. Microbiol. 31 (1993) 502–506.
[29] K.R. Caleffi, R.D. Hirata, M.H. Hirat, E.R. Caleffi, V.L. Siqueira, et al, Use of the polymerase chain reaction to detect *Mycobacterium leprae* in urine, Braz. J. Med. Biol. Res. 45 (2012) 153–157.
[30] F.B. da Rosa, V.C. de Souza, T.A. de Almeida, V.A. do Nascimento, F.G. Vasquez, Detection of *Mycobacterium leprae* in saliva and the evaluation of oral sensitivity in patients with leprosy, Mem. Inst. Oswaldo Cruz 108 (2013) 572–577.
[31] S.A. Woods, S.T. Cole, A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR, FEMS Microbiol. Lett. 53 (1989) 305–309.
[32] M. Lavania, K. Katoch, H. Singh, R. Das, A.K. Gupta, R. Sharma, et al, Predominance of three copies of tandem repeats in *rpoT* gene of *Mycobacterium leprae* from Northern India, Infect. Genet. Evol. 7 (2007) 627–631.