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Research Article

In-Vitro Cultivation of Mycobacterium Leprae and Its Confirmation by Molecular Methods

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ABSTRACT

Mycobacterium leprae was the first bacteria to be described as the causative agent of a disease by Dr. Gerhard H.Armauer Hansen in 1873. Many organisms have since been discovered and almost all have been cultivated in artificial culture media (in-vitro cultivation) except M.leprae. Numerous attempts were made to develop a synthetic artificial media for M.leprae over several years, without any success.

The present authors describe here the development of a bi-phasic artificial media (henceforth called "PRG Media") in which acid fast organisms with globi formation resembling M.leprae were repeatedly grown in liquid and solid phases between 18-22°C over 12-16 weeks. No growth was observed at 37°C till the end of incubation.

The organisms grown in the bi-phasic media were identified as M.leprae by molecular methods whereby gel images of RLEP gene showing amplified repetitive gene sequence of 129 base pair which is unique and 100% specific for M.leprae were detected in the isolates. Another molecular marker, pseudogene ML1545 which is also used for detection of M.leprae was also found to be positive using PCR.

KEYWORDS

Mycobacterium leprae, *In-vitro* culture, Biphasic media, Molecular confirmation, RLEP gene, Pseudogene.

Introduction

The discovery of Mycobacterium leprae as the causative agent of leprosy was made by Dr. Gerhard H. Armauer Hansen in Norway in 1876. It was the first organism to be labeled as the causative agent of an infectious process. Thereafter, numerous bacteria have been discovered and almost all have been grown *in-vitro* in artificially chemically defined culture media in the laboratory, except M.leprae. Thus, M.leprae is the only organism which does not fulfill the Koch's postulate till date which is essential in labeling an organism as the causative agent of a disease. All attempts to grow the bacteria in artificially culture media have failed till today [1,2]. In fact, the International Leprosy Congress in 1978 concluded that "so far there is no proof that a genuine culture of the leprosy bacilli has been obtained [3]. This holds good even today.

Due to the repeated failure of 'in-vitro cultivation' of the leprosy bacilli, research of leprosy has not progressed as expected and many facts about this disease and about the infecting agent remain unknown.

The only viable option to study M.leprae in the laboratory has therefore been to maintain the organism in '*in-vivo*' models. The first *in-vivo* model was developed by Shephard in 1960 in the mouse footpad [4]. Thereafter, Rees [5] developed the thymectomised and irradiated mouse model for *in-vivo* cultivation. Subsequently, Kirchheimer in 1974 developed the armadillo model [6]. However, each of these *in-vivo* models has their advantages and disadvantages. Thus, the only solution to the problem is to develop an '*in-vitro*' culture method like in all other bacteria, which has not been possible up till now. Once such media is developed it will be possible to ascertain the diagnosis within few days and also determine the prognosis of the disease. Different drugs can be easily tested to determine their effectiveness in every single case and newer drugs can be tested to determine their effectiveness against M.leprae.

Lastly, the development of an effective anti-leprosy vaccine would also become possible to prevent the disease amongst contacts of the patient especially children who are very susceptible to the infection. It has been postulated that if children can be kept free of the disease for the first ten years, leprosy would almost or entirely die out of an endemic country within two generations [7].

To find a solution to all the above problems, an attempt was made to develop an *in-vitro* cultivation method for M.leprae.

It was surmised by the present workers that M.leprae probably possesses some unique properties which are hindering its growth *in-vitro*.

After exhaustive literature search, it was surmised that there are two possible reasons for failure of the organism to grow in-vitro:

1. It could be due to the very slow generation time of M.leprae (average 13 – 14 days or more) which is unique in the bacterial world, as compared to E.coli with a generation time of 20 minutes.
2. It could also be that M.leprae requires an optimal temperature for growth which is lower than 37°C, which is preferred by most human pathogenic organisms. This is borne out by the successful cultivation of M.leprae *in-vivo* models like the footpad inoculation in mice and in nine banded armadillo, where the temperature is lower than 37°C. Also, the disease manifestations in humans are mainly on the exterior surfaces like skin, nose and ear lobules where the temperature is likely to be lower than 37°C as it is exposed to the environment.

Based on these unique properties of M.leprae, a bi-phasic media was developed (henceforth referred to as "PRG Media") in an attempt to cultivate the organism in-vitro.

Material and Methods

Composition of the Culture Medium (Bi-Phasic PRG Media)

The culture medium developed by PAL, RAY and GHOSH (PRG MEDIA) is a bi-phasic media consisting of a solid and a liquid phase.

- **Solid Phase:** The solid phase consisted of standard Lowenstein-Jensen media as marketed commercially.
- **Liquid Phase:** A stock solution of the liquid was prepared as follows: Middlebrook 7H9 marketed commercially (Becton Dikinson) was used.

1. 2.35gm in 450ml distilled water was dissolved and sterilized by autoclaving at 15lbs psi at 121°C for 15 – 20 minutes.
2. This sterile media was cooled to about 45°C and 1 vial of Middlebrook Growth Supplement (ADC) as available commercially (Becton Dikinson) was added aseptically to this.
3. Thyroxine sodium tablet of 50 mcg was added to the above mixture and dissolved.
4. Glycerol 2ml sterilized separately at 160 C for 1 hour in hot air oven and cooled to 45°C was added slowly to the above mixture aseptically.
5. Antifungal and antibiotic mixture was added to the above mixture as follows:
   a. Chloramphenicol – 500 mg
   b. Gentamicin – 40 mg (1 ml)
   c. Amphotericin B – 50 mg dissolved in 10 ml sterile distilled water.

The mixture is of pH-6.6

The procedure was carried out in a Safety Cabinet Level II. This constituted the Stock Liquid Media which was stored at 2°C – 8°C until further use.

Preparation of the Complete PRG Media

Stock solution of the liquid phase (2-3ml) was added to the solid phase (L.J. Media) by passing it through sterile membrane filter (0.22U) Millipore syringe type to avoid any contamination. This constituted the complete PRG Media which was stored at 2-8°C and used up within 4 – 6 weeks. This synthetic medium was used for in-vitro cultivation of M.leprae with tissue obtained from multibacillary cases of leprosy.

Selection of Patients / Collection of Material

Multibacillary cases of leprosy attending the Skin O.P.D. of M.G.M. Medical College, Kishanganj, Bihar were selected for study. Consent was taken from them for collection of the material. Permission was also sought from the Ethical Committee for carrying out the study and for collection of material from the patients. The skin over the back and forearm lesions were aseptically cleaned with alcohol and iodine application. Slit-skin smear from ear lobule and punch biopsy from skin nodules were taken. For nodules present on the body, the skin was cleaned aseptically, pinched up, and a cut was made with a sharp scalpel. The material was collected over the scalpel blade and subsequently transferred into the biphasic

culture and onto a glass slide for smear examination.

Pretreatment before Inoculation

The material thus collected was dipped in sterile normal saline in a sterile petri dish. Using sterile scissors and forceps, the skin over the tissue was carefully removed as much as possible. The tissue was then dissected in to smaller pieces (roughly 4-5 pieces). Thereafter in another sterile container, the tissues were immersed into Middlebrook PANTA (Becton Dikinson) along with Gentimicin solution, and kept for 4-6 hours. This was done to remove the skin contaminants from the tissue as much as possible. PANTA is a mixture of 5 antibiotics consisting of Polymyxin, Amphotericin B, Nalidixic Acid, Trimethoprin and Azlocillin.

Smear Examination

Material collected as above was alcohol fixed on the glass slide and stained by modified Ziehl- Neelsen's stain. Thereafter, smears were air dried, and examined under the microscope. Positive smears were graded according to standard grading method from 1+ to 6+ as per Ridley and Joplin'scheme. Those material which were 4+ to 6+ positive were selected for culture in prepared bi-phasic PRG Media in duplicate. BI was also recorded after examination of the smears.

Incubation of the Media

Collected material was teased to expose the inner content of the tissue and distributed into two halves for inoculation into separate PRG Media in duplicate. One portion was incubated at 18°C – 22°C in the B.O.D. incubator and the other half was incubated at 37°C. Incubation at both temperatures was done in a slightly slanting position so the liquid phase covered the solid phase of the media. Incubation was carried out for a total period of 12 – 16 weeks or longer if necessary up to 20 weeks.

Confirmation of Isolates by PCR Detection of RLEP Gene

Once the organisms grew in the liquid/solid media, confirmation of the isolates was done by detection of the RLEP gene. For this, isolates were sent to National JALMA Institute of Leprosy and other Mycobacterial Diseases, Agra under Indian Council of Medical Research, the reference centre for leprosy research. PCR was carried out for detection of RLEP gene by the method as described elsewhere [8,9].

Primer used for RLEP detection was as follows –

RLEP forward	5'-TGCATGTCATGGCCTTGAGG-3'	129
RLEP reverse	5'CACCGATACCAGCGGCAGAA-5'	129

Detection of Pseudogene (ML1545) and RLEP Gene

Isolates were also sent to Schefflin Institute of Health Research and Leprosy Centre (SIHR), Karigiri, Vellore, India, which is another reference centre for leprosy research. Pseudogene (ML1545) detection, which is also a specific marker for leprosy bacilli, was carried out by the method described by Chaitanya [10].

Primer used for detection of ML1545 gene was as follows –

ML1545 forward	5'-GTCCTCCGTCTTGCTGACTG-3'	112
ML1545 reverse	5'-CATACCGGCCATATTGCGTC -3'	112

RLEP gene detection was also carried out at this centre.

Results

78 cultures were put up for culture for incubation in duplicate out of which 63 grew acid fast organisms in the liquid phase kept at 18°C – 22°C. In 51 specimens, organisms grew both in the liquid and solid phase at the above temperature. All cultures kept at 37°C were negative till end of incubation.

Out of 78 patients selected for culture, 63 were male and 15 female. The age of these patients were in the group of 16-30 years and 31-45 years in case of both males and females.

Table1: Demographic characteristic of patients.

Serial number	Characteristic	Type	Number of patients
1.	Sex	Male	63
		Female	15
2.	Age	1-15 years	6
		16-30 years	29
		31-45 years	17
		45 years and above	11
3.	WHO classification	Paucibacillary	0
		Multibaccilary	78
4.	Ridley Jopling classification	Tuberculoid leprosy(TT)	0
		Borderline lepromatous(BT)	0
		Borderline borderline	0
		Borderline lepromatous	8
		Lepromatous leprosy	70
5.	Bacteriological index status	1-2	0
		2-4	49
		4-6	29
6.	Morphological index status	Atleast 70% bacilli were solidly stained (live) on smear examination	

Culture of Specimen

After examination of smears and grading, cultures were put up in PRG MEDIA as described before. Organisms first grew in about 8-12 weeks in the liquid phase at 18°C – 22°C. When the liquid phase started to show turbidity indicating growth, smears were made from the liquid phase and stained by modified Ziehl-Neelsen's method. Positive cultures showed solidly stained acid fast bacilli with globi formation (Figures 1&2). No fragmented or beaded bacilli were observed. On further incubation at 18°C – 22°C, colonies started to appear in the solid phase at about 12 – 16 weeks and thereafter grew to their full size subsequently. In few cases earlier growth in the liquid and solid phases were observed. Full grown colonies were 2-3mm in diameter, pearly white in

colour, occasionally with a buff centre (Figure 3). Colonies were rough, difficult to pick up and largely non-emulsifiable. Smears made from these colonies and stained by modified Ziehl–Neelsen's stain showed similar solidly stained acid fast bacilli with globi formation. No fragmented or beaded bacilli were observed.

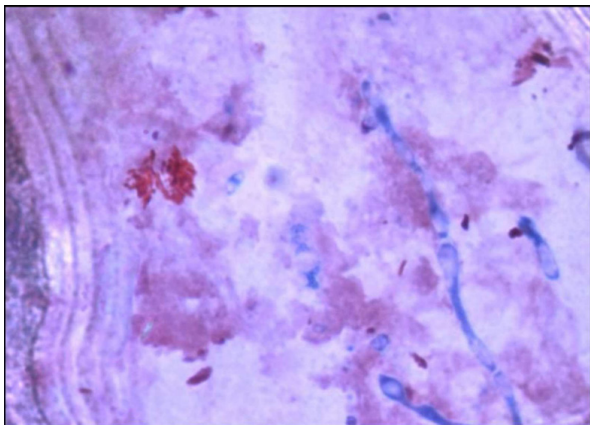

Figure 1: Z.N. Smear showing globi (Cultivated in PRG media).

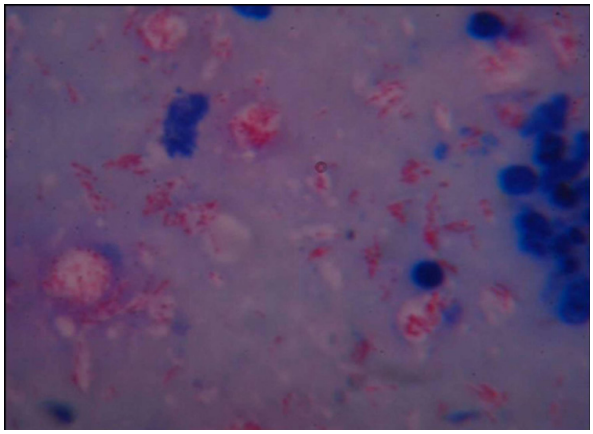

Figure 2: Z.N. Smear Examination showing AFB (Cultivated in PRG media).

Figure 3: PRG Medium showing Pearly White Colonies.

No growth was observed at 37°C in either the liquid or solid phase till end of incubation.

Further Tests

Positive cultures were sent to National JALMA Institute of Leprosy and other Mycobacterial Diseases, Agra for confirmation by PCR for RLEP gene, which is considered as positive and confirmatory marker for M.leprae.

7-8 cultures were put up for PCR test with known positive and negative controls. RLEP gene was detected with specific 129bp band on gel electrophoresis (Figure 4). The report from National Jalma Institute read as follows: -

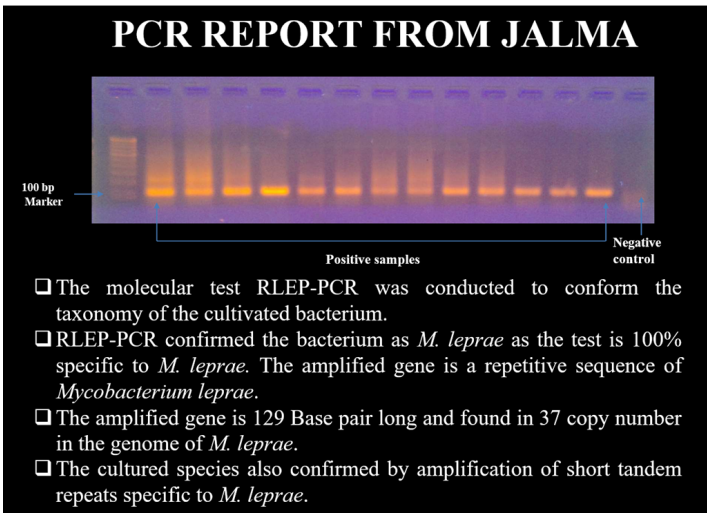

Figure 4: PCR Report from Jalma Institute.

"This amplified gene is a repetitive sequence of M.leprae and is 100% specific to the species. The cultured species is also confirmed by amplification of short tandem repeats".

No other mycobacterial species has this repeat in their genome.

Cultures were also sent to Schifflein Institute of Health Research and Leprosy Centre, Karigiri, Tamil Nadu where pseudogene detection (ML 1545) by PCR was carried out. The pseudogene (ML1545) is also considered as specific marker for M.leprae. ML1545 gene with specific 112bp was detected on gel electrophoresis (Figure 5).

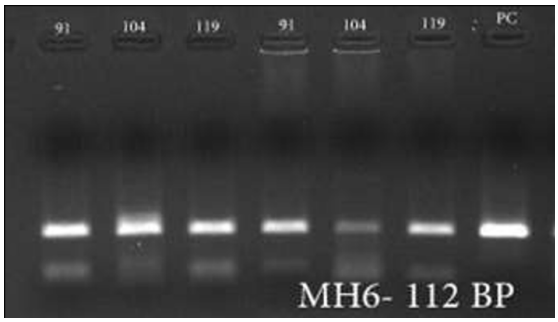

Figure 5: Pseudogene Positive for 112BP.

RLEP gene detection was also carried out at this centre and found to be present in the samples supplied.

Discussion

Various attempts have been made for over a century to grow Mycobacterium leprae in artificial culture media. But till date there is no authentic report of growing the organism in-vitro culture. The present workers hypothesized that this could be due to the slow generation time of M.leprae and also due to the fact that its optimal temperature of growth may be lower than 37°C (psychrophilic).

Two possible solutions to the problem were attempted. First was to select multibacillary cases of leprosy, where the initial count of M.leprae would be high (4+ to 6+ cases), and secondly to use a bi-phasic media whereby initial multiplication of the organism will take place in the liquid phase, and subsequently produce enough organisms to produce discrete colonies on the solid phase. Also, to reduce the generation time and to speed up the growth of M.leprae, a mixture of bacterial growth stimulants consisting of thyroxine, glycerol and Middlebrook growth supplement was added to the liquid phase.

Out of 78 samples put up for culture, 63 showed growth in the liquid phase and 51 in both the liquid and solid phase at 18 – 22°C. Failure of growth in the liquid/solid media in some cases could be due to the fact that the initial inoculum might have been low and probably required more time for growth to appear. Also, it is often seen that many patients do not give a proper history of discontinuation of treatment or having taken alternative therapy, and hence are not truly fresh untreated cases.

The bi-phasic media, developed by the present workers were also incubated at two temperatures of 18°C – 22°C and 37°C. Although distinct growth could be observed both in the liquid and solid phase at the lower temperature, no growth was observed in either phase at 37°C till the end of incubation. Bhatia [11] and Veerraghavan[7] have both noted the possibility of growing M.leprae at lower temperature from lepra tissue. Also, this fact has been borne out by the successful in-vivo cultivation of M.leprae in the footpad of mice and in armadillo, where the temperature is less than 37°C, as stated earlier.

It may be stated here that the organisms almost certainly multiplied and increased in numbers in the PRG media which is borne out by the presence of growth and colony formation on the surface of the solid media. Portaels [12] has noted that working with "difficult to grow mycobacteria" like armadillo derived mycobacteria, required very high numbers to produce one CFU (10^5 on Ogawa and 10^6 on Lowenstein Jensen). Hence, the same would be true in this case as well.

The metabolic stimulating effect of thyroxine on bacterial oxidation was first noted by Wianfan and Marx [13]. Similar effects on yeast were noted by Gutenstein and Marx [14]. Thyroid hormones act through augmented transcription of DNA, enhancing protein synthesis in cell culture and tissue culture [15]. Luxuriant growth of E.coli through effect of thyroxine has also been reported by Biswas [16]. Singh [17] also confirmed luxuriant growth of S.aureus utilizing thyroxine through radio- isotope studies using C14 labeled glucose. Biswas [18] used thyroxine solution in Dubos-L.J. media to produce limited growth from lepra tissue at 37°C. However, this temperature was found to be unsuitable by the present workers.

Glycerol is also known to stimulate the growth of all mycobacterial species except M.bovis [19]. Middlebrook ADC growth supplement is considered a growth stimulant for most mycobacterial species. Bovine albumin in ADC supplement acts as a protective agent by binding free fatty acids which may be toxic. Dextrose serves as an energy source and catalase neutralizes toxic peroxides.

The very slow growth of M.leprae in the human body and its long generation time can be explained by recent advances in genomic studies of the organism. It is now known that all functional categories of M.leprae are considerably smaller than their counterparts in M.tuberculosis. Cole [20] compared the genome of M.tuberculosis H37 Rv of 4411529 base pair which enclosed 3924 genes. As compared to this, the genome of M.leprae possesses 3268203 base pairs encoding 1604 proteins and 1116 pseudogenes. Thus, this affects mainly every part of metabolism of M.leprae due to drastic gene reduction and decay [21]. This possibly explains the failure of the organism to grow in-vitro culture.

Pseudogenes, though functionally inactive, have also been used as specific genetic and molecular markers of M.leprae. The GC ratio of M.leprae is 57.8% while that of M.tuberculosis is 65.6% [22]. Several pseudogenes have been tried for this – e.g. ML1545, ML2180, ML2179, etc. Out of these, ML1545 have been found to be present in 75.61% samples as compared with RLEP gene which is present in 58.54% cases with low BI. It has also been seen that amplification of ML1545 can detect as low as 6fg, indicating the capability of detecting DNA in at least 2 bacterial cells [23].

Confirmation of the isolates was carried out by molecular methods (RLEP and pseudogene 1545 PCR), as it is quick and very specific. For this, isolates were sent to two reference centres and both laboratories confirmed the isolates as Mycobacterium leprae by molecular methods.

Hence, it can be concluded that the organism growing in the bi-phasic PRG media both in the liquid and solid phase at a temperature between 18-20°C and exhibiting acid fast character with globi formation, a character which is present only in M.leprae, morphologically resembles M.leprae completely. Furthermore, the presence of two specific gene markers in the organism, viz. RLEP and ML1545 conclusively proves that the organism is a true representation of M.leprae and is in fact an in-vitro cultivation of M.leprae.

Hence, utilizing two unique properties of M.leprae, viz long generation time and growth at lower temperature, it may be stated

with certainty that the organisms growing in bi-phasic PRG Media at 18-22°C and M.leprae are one and the same organism.

The formulation of PRG media is under Indian Patent Application No-201631025713 dated 27.7.2016.

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References

1. Prabhakaran K. A survey of attempts at cultivation of Mycobacterium leprae in-vitro and experimental transmission of leprosy to animals. Leprosy in India. 1975; 47: 325-334.
2. Leena Das, Bijay Kumar Meher, Nikunja Bihari Das, Deepti Damayanty Pradhan, S. Pradeep, et al. A Clinco-Bacteriological Study of Leprosy in Paediatric Age Group. Advances in Infectious Diseases. 2013; 3.
3. Sanford JL, Shield MT, Root AW. Mycobacterium leprae, other mycobacteria and a possible vaccine. 1980.
4. Shepard CC. The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J Exp Med. 1960; 112: 445-454.
5. Rees RJW. Enhanced susceptibility of thymectomised and irradiated mice to infection with Mycobacterium leprae. Nature. 1966; 211: 657-658.
6. Kirchheimer WF, Storrs EH. Attempts to establish the armadillo (Dasypusnovemcinctus Linn) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int J Leprosy. 1971; 39: 693-702.
7. Veerarhaghavan N. Method of cultivation of M.leprae. Current Science. 1983; 52: 453-454.
8. Donoghue HD, Holton J, Spigelman M. PCR primers that can detect low levels of Mycobacterium leprae DNA. J Med Microbiol. 2001; 50: 177-182.
9. Yan W, Xing Y, Yuan LC. Application of RLEP gene real time PCR for detection of M.leprae DNA in paraffin embedded skin biopsy specimens for diagnosis of paucibacillary leprosy. Am J Trop Med Hyg. 2014; 90: 524-529.
10. Sundeep Chaitanya V, Das M, Eisenbach TL, Anoako A, Lakshmi Rajan, et al. Mycobacterium leprae specific genomic target in the promoter region of probable 4 alpha gluconotransferase (ML1545) gene with potential sensitivity for polymerase chain reaction based diagnosis of leprosy. Int J Mycobacteriol. 2016; 5: 135-141.
11. Bhatia VN. Effect of temperature, cholesterol and nerve tissue on multiplication of armadillo M.leprae. Ind J Lep. 1989; 61: 472-475.
12. Portaels F, Pattyn SR. Isolation of fastidiously growing mycobacteria from armadillo liver infected with Mycobacterium leprae. Int J lep. 1982; 50: 370-374.
13. Wianfan E, Marx W. Effect of thyroxine and some related compounds on bacterial oxidation. J Biol Chem. 1955; 214: 441-445.
14. Gutemstein M, Marx W. Stimulation of yeast respiration by L-thyroxine. J Biol Chem. 1957; 229: 599-602.
15. Tata JR, Widnell CC. Ribonucleic acid synthesis during the early action of thyroid hormone. Biochem J. 1966; 98: 604-620.
16. Biswas SK. Cultivation of Mycobacterium leprae in artificial culture media. Ind J Med Sci. 1989; 1: 5-10.
17. Singh KT, Ganatra RD, Shanta MS, Nimbkar YS, Gaitonde BB. Thyroid hormones and 14C glucose metabolism in bacteria. J Nucl Med. 1977; 18: 736-739.
18. Biswas SK. Effect of thyroxine on bacterial growth. Lancet. 1975; 2: 716-717.
19. Ananthanarayan, Panikar S. Textbook of Microbiology. University Press. 2013; 346.
20. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, et al. Massive gene decay in the leprosy bacilli. Nature. 2001; 409: 1007-1011.
21. Varalakshmi D, Vissa, Patrick J Brennan. The genome of Mycobacterium leprae: a minimal mycobacterial gene set. Genome Biol. 2001; 2: 1-8.
22. Singh, Puspendra, Cole S. Mycobacterium leprae: gene, pseudogene and genetic diversity: Future Microbiology. 2011; 6: 57-71.
23. Sundeep Chaitanya V, Cuello L, Das M, Sudarsan A, Pavithrra Ganesan, et al. Analysis of a novel polymerase chain reaction assay as a sensitive tool for the diagnosis of intermediate and tuberculoid forms of leprosy. Int J Mycobacteriol. 2017; 6: 1-8.