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ORIGINAL RESEARCH

Detection of *Mycobacterium lepromatosis* in patients with leprosy in India

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Introduction: The most common [illegible]d reactions in leprosy patients are type 1 reactions and erythema nodosum leprosum, with some ra[illegible] phenomenon of host response known as Lucio phenomenon or leprosy of Lu[illegible]nd Latapi which is caused by *Mycobacterium lepromatosis*. So far, no case of *M. lepro[illegible]atosis* has [illegible]en reported from India.

Materials and methods: [illegible]he main obje[illegible]ive of this study was to detect any positive cases of *M. lepromatosis* in India with suc[illegible] compli[illegible]ation. We screened slit skin smear/biopsy samples from lepromatous leprosy (LL) patients reporting to The Leprosy Mission Community Hospitals across the country. Eighty-e[illegible]ht slit s[illegible]urs were collected from leprosy patients in 70% ethanol. DNA was extracted from all t[illegible]se samples. Polymerase chain reaction (PCR) was done for 2 genes; one set was for *16S rRNA* and t[illegible] other set was for *coproporphyrinogen III oxidase (hemN)* gene. Then, sequencing [illegible]as don[illegible] for all p[illegible]sitive amplicons. Homology of the sequences was analyzed using the Basic Lo[illegible] Align[illegible]ent Search Tool at the National Center of Biotechnology Information database.

Result[illegible]: An[illegible]8 isolates, we found 4 positive cases for *M. lepromatosis*. All 4 were LL cases with a bac[illegible]riological ind[illegible] ranging from 2+ to 4+. On the basis of the National Center of Biotechnology In[illegible]rm[illegible]on Basic Local Alignment Search Tool analysis, the sequenced amplicons of both genes matched wit[illegible] *M. lepromatosis 16S rRNA* and phosphofructokinase genes but not with *hemN* gene of lepromatosis. This is the first report for the presence of *M. lepromatosis* in LL cases from India.

Co[illegible]clusion: This new species M. lepromatosis exists beyond Mexico, Singapore and it is the cause o[illegible] LL in[illegible]dia also. It may cause dual infections along with M. leprae in endemic areas like India.

[illegible]wo[illegible]ds: lepromatous leprosy, phosphofructokinase, *M. lepromatosis*, *coproporphyrinogen III oxidase (hemN)* gene, *16S rRNA* gene.

Introduction

Mycobacterium leprae has been considered to be the sole causative agent of all known forms of leprosy. The disease manifests with a broad clinicoimmunological spectrum, which has been classified by Ridley and Jopling,[1] and has been categorized into tuberculoid leprosy (TT), borderline tuberculoid leprosy, borderline leprosy (BB), borderline lepromatous leprosy, and lepromatous leprosy (LL) types. The clinical manifestation of the patient depends on the host's own immune response to *M. leprae*. Leprosy patients often, either during therapy or otherwise, tend to manifest episodes of reactions with exacerbations of existing lesions depending upon their immune status, and these have been most commonly noted as reversal reactions (type 1) and erythema nodosum leprosum reactions.

Another rare phenomenon of host response that has been noted is the Lucio phenomenon. This was first recognized by Lucio and Alvarado in 1852 and further described by Latapi and Chevez-Zamora[2] in 1948 in Mexico. It is also known as diffuse

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leprosy of Lucio and Latapi.[3] Later, Han et al,[5] while investigating the cause of death of a diffuse lepromatous leprosy (DLL) patient, isolated a *Mycobacterium* species similar to *M. leprae* from the freshly frozen autopsied liver sample of the patient and named it *Mycobacterium lepromatosis*. This mycobacterial species showed 2.1% divergence from the *16S rRNA* gene of *M. leprae*. In addition, comparison of other gene sequences belonging to *rpoB*, *16S,* and *hsp65* also confirmed a remarkable level of divergence from the corresponding sequences of *M. leprae* strains, and thereby it was assigned as a separate species.[4] The genome of this species has been recently described by Ang et al,[7] and from 126 contigs, which confirms differences at the genome level, both species were found to differ by ~13% in sequence diversity. This is in contrast to <0.005% sequence diversity observed in the global collection of *M. leprae* strains. This mycobacterial species almost has the same GC content (~57.8%) like *M. leprae,* which is significantly lower than the GC content of other mycobacterial species (60%–66%). *M. lepromatosis* is a causative agent for DLL, which carries a higher mortality rate than other forms of leprosy.[2,7–10]

Earlier, several case reports from India also showed that patients have clinical features suggestive of DLL/Lucio's Phenomenon.[9,11] However, there has not yet been reports of cases of *M. lepromatosis*, and therefore to search for the existence of *M. lepromatosis* infection we screened samples isolated from patients visiting the hospitals of The Leprosy Mission, India, from across the country. Thus far, *M. lepromatosis* has been found in patients with leprosy from Mexico, Canada, Brazil, Singapore, and Myanmar.[12–14] Recently, it was found to be a cause of leprosy, along with *M. leprae*, in red squirrels in the British Isles.[15] Thus, *M. lepromatosis* is present in the Americas, Asia, and Europe.

Materials and methods

Ethical approval

Ethical clearance for this study was provided by The Leprosy Mission Trust India Ethical Committee, under the regulations of the Indian Council of Medical Research. Written informed consent was obtained from all subjects before collection of biological samples.

Materials

In the present study, we analyzed 88 skin slit smear/biopsy samples from MB leprosy patients; the sample was collected in 70% ethanol from patients who visited The Leprosy Mission in different states of India during 2014–2017 years (Table 1).

Extraction of DNA from clinical samples

DNA was isolated from all these samples. All samples were processed for lysis in 100 µL Lysis buffer (1 M Tris-EDTA, 0.05% Tween 20, 10 mg/mL proteinase K) at 60°C for 16 hours followed by inactivation of reaction at 97°C for 15 minutes. In case of inhibitors in the lysate, it was passed through a Qiagen column (Qiagen, Hilden, Germany) before using in the polymerase chain reaction (PCR) as a template.

PCR amplification of genes targeting *16S rRNA* gene region and *hemN* and sequencing

To check the positive cases for *M. lepromatosis* in our collected samples, PCR was done for 2 sets of genes. One set was for 16S rRNA gene and other set was for *coproporphyrinogen III oxidase* (*hemN*) gene. Each PCR reaction contained 5 µL DNA, 10 µM of each primer, and Hot start Taq polymerase PCR master mix (2×) (Qiagen). The final volume of reaction mixture was made up to 20 µL with nuclease-free water. Details of primers used in this study are listed in Table 2. Amplicons of each reaction were electrophoresed through 3% agarose gel in 1× Tris borate EDTA (TBE) running buffer. Sequencing was done for all positive amplicons. Sequences obtained were analyzed using Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information database (NCBI, Bethesda, MD, USA).

PCR for *M. leprae* detection

Each PCR reaction contained 5 µL of DNA, 10 µM of each primer, and Taq polymerase PCR master mix (2×) (Qiagen). The final volume of reaction mixture was made up to 20 µL with nuclease-free water. For *M. leprae* detection, *RLEP* gene target was selected; the primer details are listed in Table 2.

PCR targeting *rpoB, folP*, and *gyrA* genes to check the drug susceptibility

We also performed PCR sequencing for drug susceptibility testing of 4 samples targeting the genes *rpoB*, *folP,* and *gyrA*. PCR-based gene amplification was done using primers according to the Guidelines of the World Health Organiza-

Table 1 Geographical distribution of patients recruited in this study

	North India	South India	East India	Central India
Total sample number 88	47	2	7	32

Table 2 List of primers used in this study

S. no	Primer sequence	Gene name	Product length	Reference
1.	5′ GTCTCTTAATACTTAAACCTATTAA 3′ 5′ CCACAAGACATGCGCCTTGAAG 3′	16S rRNA	142 bp	Han et al,[5] 2009
2.	5′ GTTCCTCCACCGACAAACAC 3′ 5′ TTCGTGAGGTACCGGTGAAA 3′	hemN	244 bp	Singh et al,[6] 2015
3.	5′ TGCATGTCATGGCCTTGAGG 3′ 5′ CACCGATACCAGCGGCAGAA 3′	RLEP	129 bp	Donoghue et al,[16] 2001

Table 3 Geographical distribution of *M. leprae* and *M. lepromatosis* detected by PCR

	North India	South India	East India	Central India
Total sample number 88	47	2	7	32
Detection of *M. leprae* by PCR	41 +ve	All +ve	All +ve	29 +ve
Detection of *M. lepromatosis* by PCR	1 +ve	All –ve	All –ve	3 +ve

Abbreviation: PCR, polymerase chain reaction.

tion's (WHO) "Global Surveillance of Drug Resistance in Leprosy 2008" for detection of mutations in *rpoB*, *gyrA,* and *folP1* genes in the *M. leprae* genome.[17]

Phylogenetic analysis

The gene sequences obtained in this study were analyzed, and additional GenBank accessions (NCBI) for 16S rRNA genes included, EU203590 for *M. lepromatosis* FJ924, GQ90 372 for Sg-1 *M. lepromatosis*, and GQ900374 for B 1 *M. matosis*. Sequence analysis was performed thro h queries to GenBank using BLAST. Phylogenetic nalysis wa er-formed using CLUSTAL W for multial nt.[20] The tree construction was done by maximum likelihood hod the Tamura–Nei model in MEGA7 soft are.[21]

Results

Origin of samples

Out of 88 samples, 47 we from North India followed by 32, 7, and 2 samples fro Cen al, East and South India, respectively (Table 1).

Detection of *M. leprae* by PCR

Initially, all samples were screened for detection of *M. leprae* by targeting *RLEP*. Among these 82 samples, only 3 were negative for *RLEP* and showed positivity for *rpoB* gene (Table 3). Out of these 4 *RLEP*-negative and *rpoB* cases, 2 belonged to Central India and 1 was from Northern India.

Detection of *M. lepromatosis* by PCR

Two gene targets specific for *M. lepromatosis* were selected – 16S rRNA gene region and *hemN* gene (Figure 1). Among all samples, we found only 4 cases positive for *M. lepromatosis*.

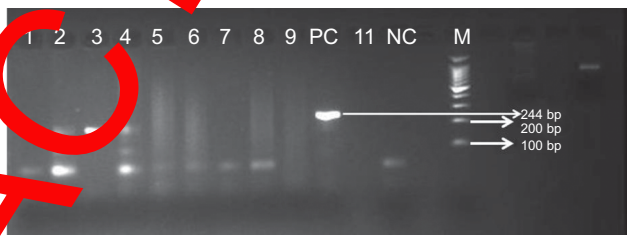

Figure 1 Gel image for *hemN* lepromatosis gene-positive samples.
ote: Product length: 244 bp.
Abbreviation: hemN, *coproporphyrinogen III oxidase*.

Positivity of these 4 samples among 88 samples constitutes 4.5% of the total cases, and the clinical details of these 4 patients are summarized in Table 3.

All 4 presented with LL with infiltration and nodules all over the body. Clinically, they were LL patients with diffuse lesions and a bacteriological index (BI) ranging from 3+ to 4+. All patients were treated according to the WHO regimen of multibacillary leprosy. In our study, we included 2 sets of genes specific for *M. lepromatosis* as reported by Han et al[14] and Singh et al.[6] We found amplifications of 142 bp and 244 bp for the 16S rRNA and the *hemN* genes, respectively. Both the genes were sequenced and found to be matching with *M. lepromatosis* in the available gene database at the time of analysis on BLAST. But at the time of analysis of *hemN* gene, we found that BLAST results matched with the gene phosphofructokinase of *M. lepromatosis* (Figure 2).

Phylogenetic analysis of samples

The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model.[22] The tree with the highest log likelihood (–1,256.25) is shown. Initial tree(s) for the heuristic search were obtained

automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measuring the number of substitutions per site (next to the branches). The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 126 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.[21] In figure 3, analysis of phylogenetic tree for *16S rRNA* showed that 3 samples CH4, M10 and M6 are closely related to *M. lepromatosis*, while 2 samples CPH570 and IAI94 were outbranched and formed a different clade with respect to *M. lepromatosis*. In phylogenetic analysis for *hemN* gene (Figure 4), no submitted sequence was found in NCBI; but in the analysis, it was clearly seen that CH7 and CPH570 samples branched from the L10 sample, which itself was a positive control of *M. lepromatosis* (nonsubmitted sequence, gifted from Dr Rahul National Hansen's Disease Programme Baton Rouge, USA); hence, CH7, CPH570, and L10 all fall into the same clade, confirming the presence of *M. lepromatosis* in these samples.

Drug susceptibility testing in patients

The drug susceptibility testing was done for the 3 patients positive for *M. lepromatosis* by 16S rRNA PCR. All patients were classified as having LL based on the Ridley–Jopling scale. Details of the patients are as follows:

Patient 1

This patient (71/F) was from Champa, Chhattisgarh, and registered in 2013. The patient had a BI of 3.3+ and had many raised skin lesions and had undergone diaminodiphenyl sulfone/Dapsone (DDS) monotherapy 40 years ago. She also had active skin lesions with thickened nerves. On analysis, the patient showed resistance to *rpoB* and *folP* with mutations at codon positions 411 (Ala–Leu) and 53 (Ala–Thr), respectively. When the sequence was analyzed with BLAST along with the lepromatosis genome, we observed a mutation at codon 54 GGG–CAG (Gln 54 Arg) but no mutation was observed in the *gyrA* gene (Figure 5).

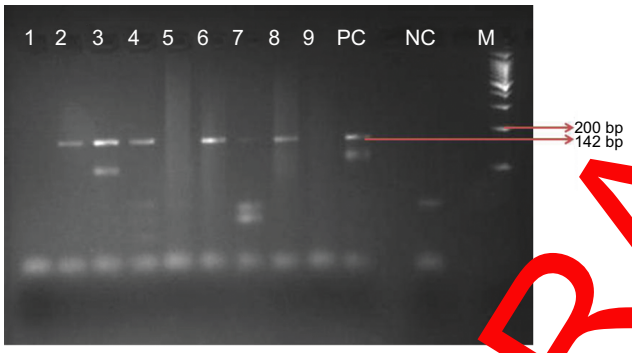

Figure 2 Gel image for 16S rRNA lepromatosis gene-positive samples.
Note: Product length: 142 bp.

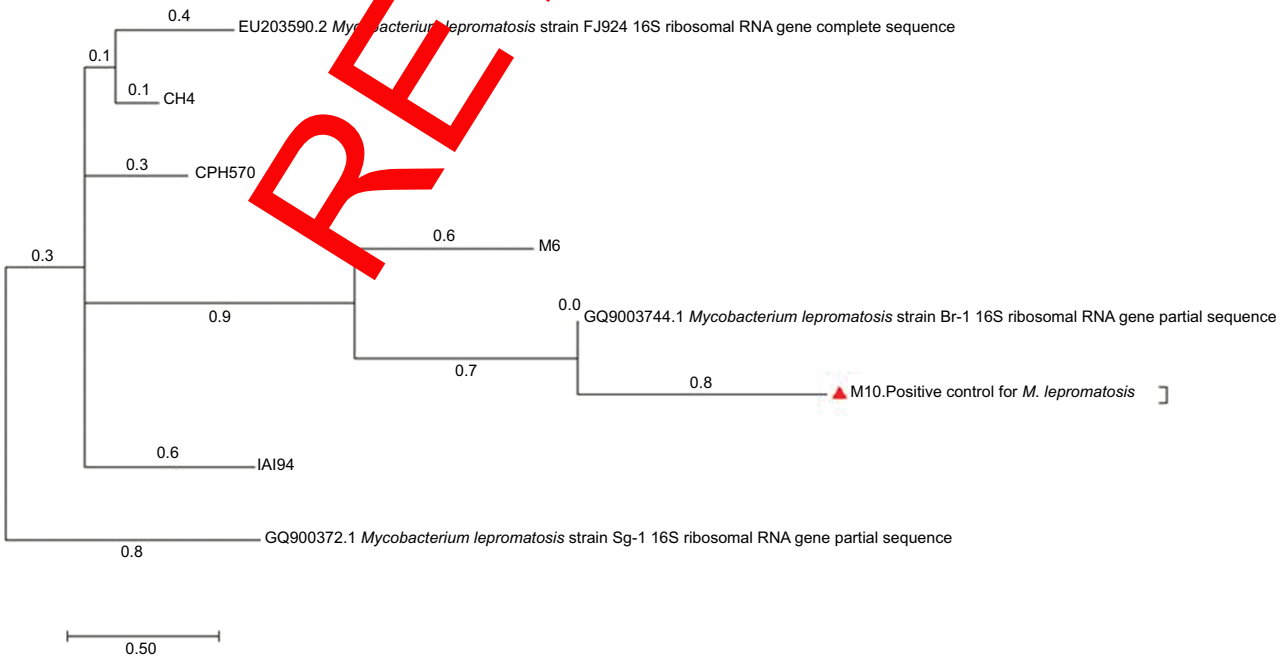

Figure 3 Molecular phylogenetic analysis by maximum likelihood method for 16S rRNA gene.

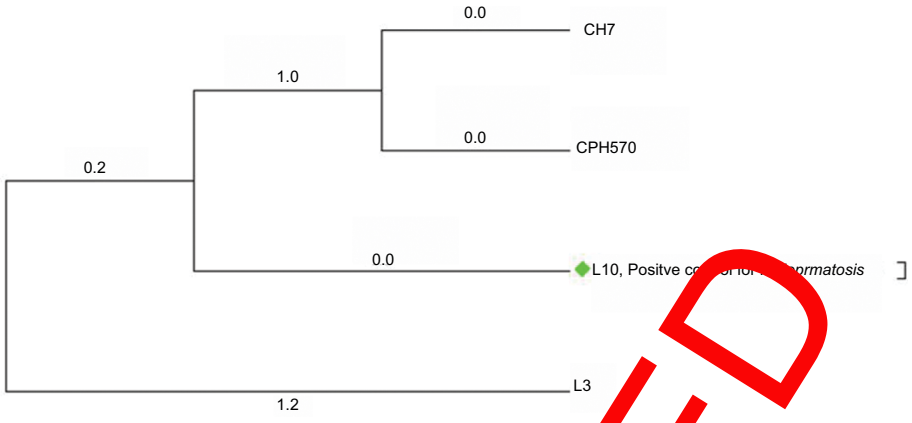

Figure 4 Molecular phylogenetic analysis by maximum likelihood method for hemN gene.
Abbreviation: hemN, coproporphyrinogen III oxidase.

Patient 2

This patient (36/M) was from Pgimer, Chandigarh, and was registered in 2015. The patient presented initially with hypopigmented patches all over the body 7 years ago. The patient presently reported with a new lesion on the face with hypopigmented patches all over the body, back, and on both hands and feet. The patient showed drug sensitivity to all 3 genes.

Patient 3

This patient (45/M) presented with multiple new lesions on face with anesthetic patches and had received multi drug therapy (MDT) for 2 years. He had a BI of 4+ at the time of sample collection. The patient showed drug sensitivity to all 3 genes.

Patient 4

This patient (30/M) was from Champa, Chhattisgarh, and registered in 2017. The patient had a BI of 4+ with infiltration nodules all over the body and had taken multibacillary/multidrug therapy (MB/MDT)/2 doses from the government Primary Health Centre (PHC) initially, but then stopped the therapy by himself 7–8 years back after feeling better.

This is the first independent confirmation of the existence of *M. lepromatosis* in Indian patients.

Discussion

Diffuse Lucio leprosy is characterized by papules, plaques, and necrotizing lesions. It was endemic to Mexico; however, it is not only restricted to Mexico, and recently this phenomenon has been reported in USA, Spain, South and Central America including Brazil, and East and West Asia.[25] Lucio phenomenon manifests 3–4 years after onset of disease and is more common in untreated patients or in those receiving inadequate treatment.[2,23] Generally, patients with extensive blisters and ulcerations representing type 2 lepra reactions have often been confused and labeled as having Lucio phenomenon.[11,24] It was also reported earlier from India that some patients showed clinical features similar to DLL/Lucio's leprosy.[11,9] Latapi and Chevez-Zamora[2] and Thappa et al[11] also warned about the improper labeling of many cases of nodular LL types as Lucio leprosy with Lucio phenomenon.

In 2008, in Mexico, a new species of *Mycobacterium* was recognized and named as *M. lepromatosis,* and this species had led to the death of 2 Mexican DLL patients.[7] In addition to Mexico, *M. lepromatosis* infection has also been reported in Canada, Brazil, Singapore, and Myanmar.[12–14] We investigated the prevalence of this newly discovered *M. lepromatosis* in 82 diffuse LL relapse cases in India. Out of these 82 cases, 3 *RLEP*-negative rpoB positive cases were from tertiary care hospitals situated in Northern and Central India (Table 1).

According to the published literature, few gene sequences belonging to *rpoB*, *16SrDNA,* and *hsp65* of *M. lepromatosis* show a remarkable level of divergence from the corresponding sequences of *M. leprae* strains.[6] WHO-recommended MDT seems to be effective in treating Lucio phenomenon. As per a case report study in India, it was observed that when MDT for leprosy is introduced at the beginning of Lucio phenomenon outbreak, the prognosis is usually good. Prognosis was noted to be poor even with proper treatment if patient presents with secondary infection and/or anemia with extensive skin involvement (a reported case study from India).[25]

In the recent past, many studies/case reports have been described with clinical presentations matching very closely those in Lucio phenomenon from other parts of the world, like Brazil and Malaysia, along with confirmation based on molecular findings.[18,19] The present study was carried out to assess the prevalence of newly discovered bacteria in DLL patients from

RNA polymerase beta subunit [Mycobacterium leprae] Alanine 411 Leucine

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Query  181    ATAGCGGCGACCACCGGACGGATATTGATCAGCGTCTGCGGCGTGATCAACTCGAC  236
              || ||||||||||||||||||||||||||||||||||||||||||||  ||||||
Sbjct  41469  ATCGCGGCGACCACCGGACGGATATTGATCAGCGTCTGCGGCGTGATCGCCTCGAC  41524

Query  1      VELITPQTLINIRPVVAAIKEFFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGLSRERA  60
              VE ITPQTLINIRPVVAAIKEFFGTSQLSQFMDQNNPLSGLTHK RLSALGP LSRERA
Sbjct  409    VEAITPQTLINIRPVVAAIKEFFGTSQLSQFMDQNNPLSGLTH RLSALGPG LSRERA  468
```

DNA gyrase A subunit Sensitive

Sequence ID: emb|CAA92430.1|Length: 1273Number of Matches: 1

Range 1: 60 to 121GenPeptGraphicsNext MatchPrevious Mat

```
Query  1      MXDSGXRPDRSHAKSARSVAETMGNYHPHGDAGIYDTLVRMAQPWSLRYPLVDGQGNFG*  60
              M DSG RPDRSHAKSARSVAETMGNYHP GDASIYDT VRMAQPWSLRYPLVDGQGNFG
Sbjct  60     MLDSGFRPDRSHAKSARSVAETMGNYHP GDASIYDTI VRMAQPWSLRYPLVDGQGNFGS  119
```

dihydropteroate synthase [Mycobacterium leprae] Alanine53 Threonine

Sequence ID: dbj|BAA84081.1|Length: 284Number of Matches: 1

```
Query  1      MIAVGAAIVDVGGESTRPGAIRTDPRVELSRIVPVVKELAAQGITVSIDTTRADVARAAL  60
              M+A GAAIVDVGGES PGAIRTDPRVELSRIVPVVKELAAQGITVSIDTTRADVARAAL
Sbjct  38     MVAEGAAIVDVGGESARPGAIRTDPRVELSRIVPVVKELAAQGITVSIDTTRADVARAAL  97

Query  61     QSGARIVNDVSGG  73
              QSGARIVNDVSGG
Sbjct  98     QSGARIVNDVSGG  110
```

dihydropteroate synthase [Mycobacterium leprae] Glutamine54 Arginine

Sequence ID: WP 04 842334.1 Length: 284 Number of Matches: 1

```
Query  1      MIAVGAAIVDVGGESTRPGAIRTDPRVELSRIVPVVKELAAQGITVSIDTTRADVARAAL  60
              M+A GAAIVDVGGEST+PGAIRTDPRVELSRIVPVVKELAAQGITVSIDT RADVA AL
Sbjct  38     MVAEGAAIVDVGGESTQPGAIRTDPRVELSRIVPVVKELAAQGITVSIDTMRADVACAAL  97

Query  61     QSGARIVNDVSGG  73
              QSGARIVNDVSGG
Sbjct  98     QSGARIVNDVSGG  110
```


Figure 5 DRS sequence alignment with Mycobacterium species.

India. For the first time, using specific DNA sequences, the presence of *M. lepromatosis* in diffuse LL has been reported in India. The finding of 4 cases with *M. lepromatosis* infection from 82 relapsed cases strongly indicates that this mycobacterial species is also prevalent in India. Kai et al[19] also reported similar findings after analysis of biopsies obtained from 19 lepromatous cases from Mexico. As the present study was limited to a few selected samples, the data is not suitable to provide data on

the actual prevalence of leprosy caused by this mycobacterial species in the country. Therefore, a widely distributed larger study needs to be undertaken to find out the actual prevalence of leprosy caused by *M. lepromatosis* in India. More cases from various countries need to be investigated to determine whether *M. leprae* and/or dual infection is the main cause of variations in clinical manifestations in leprosy.

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Disclosure

The authors report no conflicts of interest in this work.

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