Molecular Characterization of Environmental Mycobacterial Species from Leprosy Endemic Tribal Regions of North Purulia District, West Bengal

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Abstract

Background: The aim of the present study was to isolate and characterize nontuberculous mycobacteria (NTM) on Lowenstein–Jensen media supplemented with glycerol or pyruvate on two different temperatures from soil samples from leprosy endemic tribal areas of Purulia.

Methods: Mycobacterium leprae DNA was isolated from these samples followed by polymerase chain reaction (PCR) amplification using RLEP gene target specific to M. leprae. DNA was extracted from NTM cultures by lysis method. The presence of Mycobacterial DNA was confirmed by PCR using universal mycobacterial primer as 16S rRNA. NCBI nBlast was used for the authentication of NTMs, and phylogenetic tree was constructed using M. leprae and NTM species. Statistical Analysis Used: The percentile method and phylogenetic tree were used as statistical tool in this research article.

Results: The rapid-growing mycobacteria (RGM) species, 4 (80%) was obtained more than that of slow growing mycobacteria (SGM) 1 (20%) supplemented on glycerol at 30°C followed by SGM species 8 (62%) were recovered more than RGM at 37°C. Similarly, SGM species 2 (100%) were recovered on supplemented with pyruvate at 30°C and no RGM growth when supplemented with pyruvate. Further, the recovery of RGM species 3 (60%) was better on supplemented with pyruvate than SGM species at 37°C. Mycobacterium timonense was first time isolated from Indian soil samples. Highest numbers of NTM were isolated from bathing place than washing and sitting places along with M. leprae PCR positivity. Phylogenetic tree showed a close genetic evolutionary association between Mycobacterium simiae and M. leprae in the leprosy endemic environment.

Conclusion: Several NTM was isolated from soil of leprosy endemic area which might have role in susceptibility of leprosy. Phylogenetic tree revealed a closed association of M. simiae with M. leprae in the environment and might be maintaining the leprosy endemicity in north block of Purulia.

Keywords: 16S rRNA gene, leprosy, Lowenstein–Jensen media, Mycobacterium leprae, nontuberculous mycobacteria, phylogenetic tree, RLEP gene

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INTRODUCTION

Mycobacteria are environmental bacteria, and since last century, some species of mycobacteria have adapted in human as pathogen. They are ubiquitous in nature and distributed in the environment of soil and water throughout the world.1 Environmental mycobacteria are aerobic in nature, opportunistic, and acid–fast bacilli (AFB) which includes more than 198 different mycobacterial species (http://www.bacterio.cict.fr/m/mycobacterium.html accessed on July 2019), and majority of them have been isolated from the natural environment. These species belong to genus of Mycobacterium, and some species are the causative organism for disease such as leprosy, tuberculosis, Buruli

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ulcer, and pulmonary nontuberculous mycobacteria (NTM) infections. Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis*. Leprosy has existed in Middle East during biblical times and in India since Vedic times. India shares records having the highest number of new leprosy cases (135,485) of the world. The transmission of leprosy is known to occur by nasal and dermal routes.

Molecular marker such as single nucleotide polymorphisms and Variable Number of Tandem Repeat microsatellite were used for the detection of *M. leprae* from clinical and environmental sources which are useful in tracking the transmission leprosy disease in leprosy endemic areas of India. Recently, the presence of *M. leprae* has been detected in soil and water samples using 16S rRNA gene-targeted polymerase chain reaction (PCR) from the leprosy endemic regions and might be reasoned for a possible transmission of leprosy from the environment to man.

In addition, many NTM species which are considered to be nonpathogenic and have been associated with opportunistic infection in man. These NTMs may cause a variety of infections, especially in immunocompromised individuals. Recently, various species of NTM have been found to be pathogenic to human. NTM generally cause localized or disseminated infection in immune-deficient individuals. Exposure to NTM may alter the immune responses of the host and might influence the susceptibility of host to other mycobacterial diseases. There are several reports from middle east about the increasing trends of environmental NTM species, specially *Mycobacterium avium* complex (MAC) species which are pathogenic in nature and cause infection in human and might playing role as immunomodulator as providing protection or susceptibility from mycobacterial or other diseases.

Various attempts were made previously to isolate the NTM from various soil and water samples to study the distribution of NTM in environment. Several pathogenic and nonpathogenic NTM species were isolated from leprosy-endemic pockets of India. The present study was conducted to find NTM species that are present in the soil samples in tribal leprosy patient’s inhabitant areas of endemic villages of district Purulia of West Bengal. The isolation of NTM on Lowenstein–Jensen (LJ) medium supplemented with glycerol or pyruvate was observed on two different temperatures at 30°C and 37°C, respectively. Isolated NTM cultures were confirmed by Ziehl–Neelsen (ZN) method. PCR amplification of mycobacterial DNA was performed using 16S rRNA gene target, and confirmation of *Mycobacterium* genus was performed by PCR sequencing method. *M. leprae* DNA was isolated from soil samples and confirmed by PCR using RLEP gene target. The phylogenetic tree was constructed to find the genetic distance of *M. leprae* with NTM species in leprosy-endemic environment.

![Figure 1: Map of Purulia and prevalence of leprosy](image)

**METHODS**

**Site of sample**

Two leprosy-endemic districts (Arsha and Jhalda) of Purulia tribal region were selected as described earlier [27] [Figure 1]. Soil samples were collected from the banks of ponds used by leprosy patients for their daily washing and bathing and other activities [Figure 2]. In addition to the daily washing and bathing, the pond water was also used for cooking, drinking, and animal washing purposes. Thus, the sanitation of leprosy patients was very poor, as the stationary pond water was being used for all domestic purposes.

**Sample collection**

Soil samples were collected after digging soil (3–4” deep) and were transferred in clean plastic containers (10 g each) with the help of a “trowel” and the containers were capped tightly to avoid any cross-contamination. The samples thus obtained were labeled with village name and location site code. A total 50 soil samples were collected from different inhabitant places such as bathing area (20), sitting area (15), and washing area (15) of leprosy patient’s families. The collected soil samples were transported within 2 days to laboratory at room temperature (RT) (18°C to 20°C) and kept at 4°C until further processing for isolation of mycobacteria.

**DNA extraction from soil samples**

*M. leprae* DNA was isolated from the soil samples using DNeasy PowerSoil Kit (Qiagen, Catlog. No. 1288-50). Briefly, soil samples were dissolved in sterile distilled water (50 mL) and were centrifuged at 2000 × g for 5 min. The supernatants were collected in 50 mL sterile tubes and centrifuged again at 10,000 × g for 30 min. The supernatants were discarded and pellets were weighed (0.25 g), dried, and were taken in 1.5 mL microfuge tubes and were followed by soil DNA extraction kit protocol. Samples (0.25 g) were added to the Power bead (Ceramic 1.4 mm) tubes provided in the kit and vortexed properly. 60 µl of solution C1 was added to the tubes and vortexed (Spinex) for 10 min. Microcentrifuge tubes were centrifuged again at 8000 × g for 30 s at RT, and supernatants were transferred to 2 mL new microcentrifuge tubes. 250 µl of solution C2 was added to each supernatant and was vortexed followed by incubation at 4°C for 5 min.
Microcentrifuge tubes were centrifuged again at 8000 × g for 1 min at RT. The supernatants (600 µl each) were transferred to 2 mL microcentrifuge tubes. 200 µl of solution C3 was added to each supernatant and vortexed carefully followed by incubation at 4°C for 5 min. Microcentrifuge tubes were centrifuged at 8000 × g for 1 min at RT. The supernatant was transferred to a 2 mL microcentrifuge tube, and 1.2 mL of solution C4 was added to each supernatant and vortexed again for 5 s. Approximately 675 µl each was loaded onto a spin filter column and centrifuged at 8000 × g for 1 min at RT. Flow through was discarded, and the remaining mixture was added to the spin filter column. 500 µl of solution C5 was added to each and centrifuged at 8000 × g for 30 s at RT. Each spin filter was kept in a clean new 2 mL microcentrifuge tube, and 100 µl of solution C6 was added to the center of the white filter membrane and centrifuged at 8000 × g for 30 s at RT. DNA was stored in −20°C for downstream processing.

Mycobacterium leprae DNA amplification from soil samples

PCR amplification of M. leprae DNA was carried out in 20 µl of reaction volume containing 3 µl of the template. The primers (PS2) reverse and forward (PS1) were used in the final concentration of 0.25 µM each with 1X QIAGEN Mix. M. leprae-specific RLEP primers sequences (PS1 5′-TGC ATG TCA TGG CCT TGA GG-3′ and PS2 5′-CAC CGA TAC CAG CGG CAG AA-3′) were used as described earlier.[28] PCR amplification consisted of first stage of single cycle of denaturation at 95°C for 5 min followed by second stage of 35–40 cycles of 30s at 94°C, 30s at 58°C, and 1 min at 72°C and final stage of single cycle of 10 min at 72°C. PCR reaction without DNA was used as negative control (reagent control). Purified M. leprae DNA (Thai strain, BEI Resources, USA) was used as positive control.

Decontamination and processing of soil samples

Protocol: soil samples (5 g each) were dissolved in 50 ml of distilled water and samples were centrifuged at 2000 × g for 5 min; turbid supernatants were transferred into new tubes and centrifuged again at 10,000 × g for 15 min. The supernatants were discarded, and pellets were treated with 3% sodium dodecyl sulphate (SDS) and 4% NaOH. Further, the mixtures were divided in 2 Parts A and B and incubated for 15 min and 30 min, respectively. Then, both the parts of samples were centrifuged to obtain pellets, and the supernatants were discarded. The pellets were treated with 2% cetrimide solution and incubated for 5 min for rapid-growing mycobacteria (RGM) and 15 min for slow-growing mycobacteria (SGM). After incubation, sediments were washed twice with distilled water. 100 µl of distilled water were added in the sediments, and the decontaminated suspensions were added on LJ medium. Slants were incubated at 30°C and 37°C, and growths were observed from 7 days to 1 month period. The primary colonies, if noted, were subcultured on LJ medium again. The presence of Mycobacterium genus was confirmed by ZN staining from single colonies obtained from LJ slant.

Isolation of nontuberculous mycobacteria on glycerol and pyruvate supplemented Lowenstein–Jensen medium

It is known that glycerol serve as a carbon source which is favorable to the growth of the human type tubercle bacillus while being unfavorable to the bovine type. On the other hand, pyruvate incorporates pyruvic acid into the LJ basal medium to stimulate the growth of Mycobacterium bovis and mycobacteria spp. other than Mycobacterium tuberculosis.

Approximately 0.5 ml of the pellet from each processed sample was inoculated in duplicate onto LJ media supplemented with glycerol or pyruvate. Further, the inoculated culture bottles (McCarteny bottle) were incubated at two different temperatures, 30°C and 37°C for four to 6 weeks in a vertical position which lowers the development of individual colonies. During the inoculation, the slants were observed weekly for any growth of mycobacteria.

Morphological observation and identification of the nontuberculous mycobacteria isolates

The LJ slants showing growth within a week were considered RGM, i.e., confirmed by morphological examination of colonies and ZN staining. Colonial morphology of RGM was observed either as pale colour, small off-white, or cream coloured or finely wrinkled and creamy white color or turning from white to a nonpigmented creamy yellow colour. The LJ slant showing growth after 2–6 weeks was considered SGM. Colonial morphology of SGM was observed as either smooth, rough, and nonpigmented colonies or yellow pigmented colonies, and yellow to orange pigmented colonies when grown even in darkness. The NTM culture isolates were subjected to ZN staining for the confirmation of the presence of AFB and PCR method for the confirmation of NTM species.

Genomic DNA extraction from nontuberculous mycobacteria culture

DNA was isolated by scraping all the visible growth from LJ slant as described earlier.[21] Briefly, the NTM growth was collected in 400 µl of 1x Tris-EDTA (T.E) buffer, pH 8.0, and was kept in water bath at 95°C for 15 min followed by incubation at −80°C for 10 min. Lysozyme (10 mg/ml) was
added in each tube and incubated at 37°C for 2 h. Proteinase K (10 mg/ml) and 10% SDS were added to each sample tube and vortexed thoroughly followed by incubation at 60°C for 1 h. A mixture of 5M cetyltrimethylammoniummmonium bromide was added to each sample tube and each tube was vortexed vigorously until the suspension turned hazy followed by incubation at 60°C for 20 min. Chloroform–isoamyl alcohol (24:1) was added to each tube followed by vortexing and centrifugation at 8000 × g for 10 min at room RT. Aqueous phase containing the genomic DNA was transferred to a new clean microcentrifuge tube, and ice cold isopropanol was added for precipitation of DNA. Each sample was kept at −20°C for overnight. Sample was centrifuged at 8000 × g for 15 min, and supernatant was discarded. The pellet was washed with 70% ethanol by centrifuged at 8000 × g for 15 min. Supernatant was discarded, and pellet was kept for drying at 37°C for 2–3 h. Pellet was finally suspended in 100 µl TE buffer and stored at −20°C for further downstream analysis.

Polymerase chain reaction amplification nontuberculous mycobacteria DNA targeting 16S rRNA gene

PCR amplification was carried out in a total 20 µl of reaction volume that contained 3 µl of template DNA, primers at final concentration of 0.25 µM (forward and reverse), and 1X Taq PCR master mix (Qiagen India). Mycobacterial-specific 16S rRNA primers (P1: 5'‑AGAGTTTGATCCTGGCTCAG−3' and P3: 5'-CCTGACCCGAAAAGCTTTCC-3’) described earlier were used.[29] PCR reaction without DNA (reagent control) was used as negative control. Mycobacterium smegmatis DNA was used as positive control in the reaction mix. The amplification was carried out in a thermal cycler (Corbett Research) under the 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, annealing at 60°C for 2 min, and extension at 72°C for 4 min with a final extension at 72°C for 10 min. PCR product containing amplified fragment of the target region was electrophoresed in a 2% agarose gel (Sigma) using Tris-Borate-EDTA buffer at 100 volts constant voltage.

Polymerase chain reaction sequencing analysis of nontuberculous mycobacteria and phylogenetic tree

PCR amplicons were outsourced at Eurofins Genomics India Pvt Ltd Delhi. Sequences were compared with the NCBI nucleotide database using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the confirmation of NTM species. The phylogenetic tree was constructed using all NTM species along with M. leprae sequences by Mega X software, Pennsylvania State University, USA.

RESULTS

Detection of mycobacteria by Ziehl–Neelsen staining

The presences of AFB in 25 growths on LJ medium were confirmed by ZN staining. AFBs were seen as pink rods in shape.

Growth of nontuberculous mycobacteria on Lowenstein–Jensen media

Lowenstein–Jensen media-enriched glycerol

Fifty soils samples were processed and inoculated on LJ media supplemented with glycerol followed by incubation at two different temperatures (30°C and 37°C). Higher number, 13 of 50 (26%) of NTM isolates were recovered at 37°C than NTM isolates at 30°C, 5 of 50 (10%). Further, higher number of RGM 4 of 5 (80%) isolates were obtained than SGM isolates 1 of 5 (20%) at 30°C. On the other hand, higher number of SGM 8 of 13 (62%) isolates were recovered at 37°C as compared to RGM isolates, 5 of 15 (38%) at 37°C [Figure 3a].

Lowenstein–Jensen media-enriched pyruvate

Fifty soils samples were processed for growth on LJ media supplemented with pyruvate followed by incubation at two different temperatures (30°C and 37°C). Out of these 7 isolates, only 2 (29%) were SGM at 30°C without any growth of RGM. On the other hand, 5 isolates (71%) were obtained than SGM isolates 1 of 5 (20%) at 30°C. On the other hand, higher number of SGM 4 of 5 (80%) isolates were grown at 37°C, of which 2 (40%) were SGM and 3 (60%) were RGM at 37°C [Figure 3b].

Polymerase chain reaction amplification and sequencing method

PCR method analysis revealed that all 25 samples were positive for mycobacterial DNA, and amplicons were visualized on 2% gel electrophoresis. The M. smegmatis DNA was used as positive control in PCR method and compared with all PCR amplicons. The PCR amplicons were outsourced for commercial sequencing from Eurofins Genomics India Pvt. Ltd. The sequences generated after sequencing were compared with the mycobacterial sequence database present in NCBI using BLAST [Figure 4].

Figure 3: (a) Isolation of nontuberculous mycobacteria species on Lowenstein–Jensen media supplemented with glycerol at 30°C and 37°C. (b) Isolation of nontuberculous mycobacteria species on Lowenstein–Jensen medium supplemented with pyruvate at 30°C and 37°C.
Nontuberculous mycobacteria species grown on Lowenstein–Jensen media supplemented with glycerol at 37°C

Of 13 NTM isolates, 8 (62%) belonged to SGM category, and PCR confirmation showed 2 (25%) of *Mycobacterium simiae* species, isolated from bathing areas (TCSL 8 and TCSL 9) of Jhalda region. 2 (25%) *Mycobacterium scrofulaceum* isolates were recovered from sitting area (TCSL 6) and washing area (TCSL 10) of Jhalda region. Further, 2 (25%) *Mycobacterium parascrofulaceum* species were isolated from bathing areas (TCSL 2 and TCSL 3) of Arsha region followed by 1 (12.5%) *Mycobacterium europaeeum* isolated from washing area (TCSL 4) and 1 (12.5%) *Nocardia* spp. as nonchromogen isolated from bathing area (TCSL 14) of Jhalda region.

Of 13 NTM isolates, 5 (38%) NTM culture belonged to RGM category and PCR confirmation showed as 2 (40%) as *Mycobacterium fortuitum* isolated from bathing areas (TCSL 1) of Arsha region and sitting area of (TCSL 6) Jhalda region. 1 (20%) *M. smegmatis* species was isolated from washing areas (TCSL 4) of Arsha region and other 2 (40%) NTM species observed as *M. spp.* isolated from washing area and (TCSL5) and sitting area (TCSL 11) of Jhalda region [Figure 3a].

Nontuberculous mycobacteria species grown on Lowenstein–Jensen media supplemented with glycerol at 30°C

Of 5 NTM isolates, 3 (60%) NTM cultures belonged to RGM category and PCR confirmation showed as 2 (40%) as *Mycobacterium chimaera* species isolated from bathing areas (TCSL 4) of Arsha region. Other 4 isolates (80%) belonged to RGM category and PCR confirmation showed 2 (50%) as *Mycobacterium timonense* species belonged to bathing areas (TCSL 1) of Arsha region and washing areas of (TCSL 5) Jhalda area. *M. timonense* was isolated for the first time from soil samples in the present study. Other 2 (50%) of isolates of RGM were confirmed as mycobacterial species obtained from sitting area (TCSL 6) and washing area (TCSL 10) of Jhalda region [Table 1].

Nontuberculous mycobacteria species grown on Lowenstein–Jensen media supplemented with pyruvate at 37°C

Of 5 NTM isolates, 3 (60%) NTM cultures belonged to RGM category and PCR confirmation showed 1 (33%) *M. timonense* species isolated from washing areas (TCSL 5) of Jhalda region followed by 2 (66%) *M. spp.* obtained from washing area (TCSL 4) and bathing area (TCSL 8) of Arsha region [Figure 3b]. Of 5 NTM isolates, 2 (40%) NTM cultures belonged to SGM category and PCR confirmation showed these as *M. spp.* and *Nocardia* obtained from bathing places (TCSL 3, 9) of Arsha and Jhalda regions.

Nontuberculous mycobacteria species grown on Lowenstein–Jensen media supplemented with pyruvate at 30°C

Two NTM cultures belonged to SGM category, and PCR confirmation showed 1 (50%) *Mycobacterium intracellulare* species, isolated from bathing areas (TCSL 3) and 1 (50%) *Nocardia* spp. obtained from washing areas (TCSL 10) of Arsha region.

Polymerase chain reaction amplification using RLEP gene region of *Mycobacterium leprae*

PCR using RLEP region (129 bp) specific to *M. leprae* genus was performed from all soil samples. PCR amplicons were visualized on 2% agarose gel. The PCR positivity of *M. leprae* DNA was noted in 18 of 50 (36%) from soil samples. Further, *M. leprae* DNA PCR positivity was observed as 9 of 20 (45%)
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in bathing place, 6 of 15 (40%) in washing place, and 3 of 15 (20%) from sitting place [Table 2].

Phylogenetic tree of Mycobacterial species
The 16S rRNA gene sequences of all the mycobacterial species were aligned using Mega X software, and the evolutionary hierarchies of mycobacterial species were constructed using the Neighbor Joining method [Figure 5]. The phylogenetic trees showed that M. leprae was present in close genetic evolutionary relationship with M. simiae species. Other NTM species including M. fortuitum, M. scrofulaceae and M. smegmatis was found in the adjacent nodes of the phylogenetic tree while M. chimaera, M. intracellulare, M. timonense, M. europaeum and M. parascrofulaceae were distantly related with each other.

Discussion
India has the largest tribal population after Africa in the world. A substantial list of scheduled tribes in India is recognized as tribal under the Constitution of India. According to the 2011 census, tribal people constitute 8.2% of the nation’s total population equivalent to almost over 104 million people. Purulia is the oldest known districts in West Bengal. These tribal people basically occupy the regions of forest and hilly areas. Tribal population is considered to be the most backward and deprived section of the Indian society. Socioeconomic development of these tribal people has been neglected from British period to the present age of globalization. These aboriginals have a unique lifestyle of their own and are untouched by the modernity. In the era of development and globalization, they still face the problems of illiteracy, vicious poverty, ill health, poor livelihood, and low income which force them to live in primitive conditions. Purulia is one of the most backward tribal districts in the country. Tribal of this district have distinct age-old social system, cultural tradition, customs, values, lifestyle, and languages. However, in terms of economic condition, they can be considered to be the poorest of the poor in the country. Tribals of this district use the forest as a means for their sustenance (www.censusindia.gov.in). Leprosy is endemic in certain parts of India and accounts for 63% of world leprosy population.[4] Prevalence rate (PR) of Purulia is much higher than the elimination figure of <1/10,000 and was last recorded as 3.55/10,000 population. Recently, it was noted that PR of leprosy of Arsha and Jhalda was 2 and 1.8 respectively.[27] Soil samples were collected from bathing,
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was also isolated from washing area of Arsha region, and it was also reported from Purulia region earlier. [25] On the other hand, more number of NTM isolates supplemented with glycerol was observed at 37°C which belonged to SGM categories such as M. simiae, M. scrofulaceaeum, and M. europaeum species.

Overall, NTM isolation on LJ medium supplemented with glycerol and pyruvate was highest at 37°C than NTM isolation at 30°C. Further, highest number of RGM 80% was recovered on with glycerol at 30°C. The growth of RGM was more on with glycerol and might be due to the availability of glucose in the glycerol supplement. Similarly, only SGM 100% isolates were grown on with pyruvate at 30°C which were confirmed by PCR sequencing method. NTM species were observed as M. intracelullare species isolated from bathing area of Arsha region. It was also noted that other MAC belonging species such as M. intracelullare and M. chimaera isolated from bathing and washing places of pond area of leprosy patients are known to be pathogenic in nature and might help in making people susceptible toward infectious disease like leprosy by modulating the host immunity. [11] Further, highest numbers of SGM 62% were isolated on LJ medium supplemented with pyruvate at 37°C from bathing area of Arsha region followed by 38% RGM isolation at 37°C from washing area of Jhalda region. The PCR sequencing confirmation of SGM isolates was found to be as M. simiae, M. scrofulaceaeum, and M. europaeum and RGM were M. fortuitum and M. smegmatis species. Earlier, several scientists isolated NTM species from clinical and environmental sources from different parts of India. Most frequently isolated NTM species from South India was Mycobacterium avium intracelullare (MAI) from south India. [29] Further, scientist isolated MAI and M. scrofulaceaeum in water and dust. [21] It was shown that NTM were isolated including M. fortuitum, M. avium, Mycobacterium kanssaii, Mycobacterium terrae, and Mycobacterium chelona in water and M. avium, M. terrae, and M. chelona in soil samples from leprosy endemic regions of Ghantampur, Uttar Pradesh. [24]

The highest (45%) M. leprae DNA PCR positivity was obtained from bathing places along with NTM such as M. timonense, M.
fortuitum, M. parascrofulaceum, M. intracellulare, M. simiae, and Nocardia species. Second, 40% M. leprae DNA PCR positivity along with M. timonense, M. chimaera, M. europaeum, M. smegmatis, M. scrofulaceum, Nocardia, and M. spp. were noted from washing places. Recently noted, Mycobacterium gilvum and M. parascrofulaceum were isolated from bathing area water samples of leprosy patients from Purulia.25,26

Finally, 20% M. leprae DNA PCR positivity along with M. fortuitum, M. scrofulaceum, and M. spp were noted from sitting area [Table 2]. Thus, it is noted that viable M. leprae is always present in the leprosy-endemic environment along with various cultivable NTMs. Whether these NTM species are providing a niche for M. leprae to remain viable in the environment for long time is not known. Further, the phylogenetic tree was constructed and close evolutionary relationship was observed between M. leprae and M. simiae. Likewise M. fortuitum, M. scrofulaceum, M. smegmatis was found in adjacent nodes of phylogenetic tree while M. chimaera, M. intracellulare, M. timonense, M. europaeum and M. parascrofulaceum were distantly related with each other. Increasing trends of newly diagnosis NTM species and improvement in various detection method using culture and molecular marker might be useful for immediate treatment and management of NTM as opportunistic organism in other diseases. Their presence probably provides an evolutionary drive toward development of immune system or susceptibility toward leprosy in human.23

Conclusion
The isolation of RGM was more on with glycerol at 30°C as compared to SGM at 30°C. SGM was recovered more on with glycerol than RGM species at 37°C. On the other hand, the isolation of SGM was more on with pyruvate at 30°C, and no growth of RGM was observed at 30°C. The growths of RGM were more on with pyruvate at 30°C as compared to SGM. The presence of M. leprae and several NTM were observed in bathing places followed by washing places and sitting places. Further, the phylogenetic tree was constructed and showed that M. simiae which is a known opportunistic pathogen might be responsible for maintaining the viability of M. leprae in the environment and maintaining the leprosy endemicity in North block of Purulia.

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Conflicts of interest
There are no conflicts of interest.

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