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Original Research Article

Detection of Mycobacterium Leprae in Environmental Samples Collected from Residence Area of Patients with Active Leprosy by Targeting RLEP-PCR – A Prospective Analytical Study from Central India

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Abstract:

Introduction: The transmission mechanism of *Mycobacterium leprae* is not clearly known. There are evidences available showing healthy individuals living in close contact with active leprosy patients are at high risk. In studies from India itself, *Mycobacterium leprae* DNA, have been isolated from environmental samples such as soil and water which shows shedding of organism from active leprosy cases. The possibility of transmission of infection from environment being as a source of infection has yet to be proven. These findings challenged the long-held belief that *M. leprae* transmission occurred solely through human-to-human contact (respiratory route like in Tuberculosis).

Aim/Objective: The central aim of this investigation was to detect *Mycobacterium leprae* DNA in environmental samples like fomite (pillow cover/ towel/ bedsheet etc.) by targeting the RLEP sequence using Polymerase Chain Reaction.

Methods: This study is a prospective analytical study done for a study period of six months at an institute of national importance situated in Raipur, Chhattisgarh, India in which newly diagnosed patients of active leprosy disease attending the institute during study period were registered and samples from their household were collected. A total 147 sample were collected from 29 cases. DNA was extracted using salting out – proteinase K method. Detection of *M. leprae* was done using RLEP-PCR.

Result: A total of 63 water samples and 84 fomite swab samples were collected from the houses of 29 patients and from various water bodies in and around their residence area. In this study, we analyzed 84 fomite samples, including swabs from beds, towels, and door handle, using LP-1 and LP-2 primers to amplify a 129 bp RLEP sequence. Among these, one door/floor sample and one towel sample from the household of a multibacillary (MB) case tested positive for RLEP-PCR. All remaining fomite samples, as well as all 63 water samples, were PCR negative.

Conclusion: The detection of *Mycobacterium leprae* DNA in two fomite samples (door/floor and towel) from the household of a multibacillary case suggests the shedding of bacilli from the active case. However, *M. leprae* DNA were absent in all water samples and the majority of fomite samples.

Keywords: Leprosy, Environmental health, Transmission, Molecular Biology, RLEP-PCR.

Introduction

Till date exact mode of transmission of leprosy is not known; however, from studies it has been speculated that active transmission of leprosy occurs by aerosols especially from a highly infected human source, some studies have suspected transmission from ulcers of leprosy patient to abraded skin of normal healthy individual suggesting skin to skin transmission. Some of the very old studies have also suggested transmission by vectors (insects). Many recent studies showed presence of *Mycobac-*

terium leprae in environmental sources viz. soil and water.[1-3] Hence Public health Leprosy control activities all around the world focused on early diagnosis and treatment of active leprosy case by chemotherapy i.e. MDT. So far, this strategy has only helped in controlling the disease in community up to a certain level; No country has been eradicated leprosy like polio and other infectious disease so far. This calls for detailed investigation for transmission dynamics of Leprosy. The role of soil and water in leprosy transmission is only a hypothesis which is yet to be proved by scientific experiments but if this is proven we need to develop new strategy to eradicate this crippling disease from earth.

In highly endemic area for leprosy, not only household contacts of seropositive patients, but also people living in the vicinity of a seropositive patient are more likely to harbor antibodies against *M. leprae*.[4] Indirect evidence of this theory seems true from the fact that in the recent past, contaminated water supply systemshave been responsible for several hospital and community outbreaks of mycobacterial infections. [5] Researchers have shown presence of *M.leprae* may in free living amoeba and in Entamoebacysts in water samples. [6] Some other old research studies show presence of *M.leprae* in insects, fish, plants and animals like armadillo. [1,7-9]Other studies shows that leprosy is not a disease occur by single exposure and continuous exposure for long period is required along with many other factors like immunity of the susceptible host which in turn results in varied clinical presentations at the time of diagnosis.[10].

The Government of India has launched National Strategic Plan (NSP) & Roadmap for Leprosy (2023-27), to achieve zero transmission of leprosy by 2027 i.e. three years ahead of the Sustainable Development Goal[11]. But there are very few studies being done to monitor transmission of leprosy from active cases in current scenario. Determination of the chain of transmission is very important to prevent occurrence of new cases. Our study will initiate a hypothesis whether lepra bacilli shedding is there in fomite/ environment.

Aim/Objective: Therefore, the primary objective of this study was to detect *Mycobacterium leprae*DNA in fomite/environmental samples collected from the residential areas of patients with active leprosy using PCR targeting the RLEP gene sequence.

Material and Method

This study was aprospective analytical study done for a study period of six months i.e. at Institute of National importance in Raipur, Chhattisgarh in which newly diagnosed patients of active leprosy disease attending the institute during study period were registered. Proper informed consent was taken from each case so as to collect environmental samples including fomites and water sources from vicinity of each active case household.

Sample Collection: Water samples were collected from waste pipe and potable water from patient's house and neighboring water source. Swabs from fomite samples like door handles, used pillow cover, used bed sheet and used towel were collected.[12]

Collection of water sample: 5-10 ml of water sample were collected in sterile container from waste pipe of bathroom/ gutter and from potable water.[12]

Collection of Fomite sample: one sterile cotton swab each for each area was dipped in Tris- EDTA (TE) buffer. Excess solution was squeezed and swab was rubbed on (one separate swab for each fomite) door handle, pillow cover, towel and bed handle. Samples were transported immediately to institute laboratory and processed for DNA extraction. *M.leprae*was detected by RLEP PCR set up by method already defined .[3]

DNA Extraction from water sample: One milliliter water sample was pipetted in centrifuge tube and centrifuge at 9500g for 15 min& supernatant was discarded. Pellet was dissolved in 500µl of 0.125M EDTA (pH 8.0), incubated at 50°C for 4-5 hrs in water bath, centrifuged at 9500g for 10min. & supernatant was discarded. 100µl lysis buffer (proteinase K and Tween 20) was added in pellet & incubated at 60°C overnight in a water bath. Proteinase K reaction was terminated at 95°C for 10 min. 30µl of 10% SDS was added & incubated at 60°c for 1 hr in water bath. Then 500µl of TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl and 1% Polyvinyl polypyrrolidone) were added& kept for 1hr at room temperature with vortex and mixing multiple times. Centrifugation was done at 850g for 10 min & supernatant was collected. Precipitation of nucleic acid by isopropanol 0.6 volume of isopropanol was added in each tube and incubated at -20ºC for overnight for DNA precipitation. Tubes were centrifuged for 15 minutes at 9500g. After centrifugation supernatant of each tube was discarded. Washed with chilled 70% ethanol, each tube 150µl ethanol was added. Tubes were again centrifuged at 9500g for 5 minutes. After centrifugation supernatant of each tube was discarded and tubes containing DNA were air dried, dissolved in 40µl of T.E. buffer and stored at -20ºC.[13]

DNA Extraction from fomite sample: The fomite samples collected by swab and put in 200 µl TE buffer was vortexed and swab was taken out. The Suspension was boiled for 5-10 minutes and then snap chilled in ice. This makes the cell wall rupture. 80µl of lysozyme (20mg/ml) was added in each tube and incubated at 37ºC for 2 hours in

shaking water bath. 80µl of 10% SDS and 10µl of proteinase k (10mg/ml) was added in each tube and mixed by vortex followed by incubated at 65ºC for 30 minutes in shaking water bath. 100µl of CTAB and 80µl NaCl was added in each tube. Vortex and incubated at 65ºC for 30 minutes. Equal volume (645µl) of chloroform and Isoamyl alcohol mixture (24:1) was added in each tube centrifuged at 9500g. For 5 minutes. Protein and carbohydrate were settled down and supernatant of each tube was transferred in another respective tube. 0.6 volume of Isopropanol, 180µl was added in each tube and incubated at -20ºC for overnight for DNA precipitation. Next day, tubes were centrifuged for 15 minutes at 9500g. After centrifugation supernatant of each tube was discarded. In each tube, 150µl of chilled 70% ethanol was added by side wall for washing of DNA. Tubes were again centrifuged at 9500g for 5 minutes. After centrifugation supernatant of each tube was discarded and tubes containing DNA were air dried, dissolved in 40µl of T.E. buffer and stored at -20ºC.[14]

Amplification using *M leprae* specific RLEP region: RLEP PCR amplification using the *M. leprae*-specific repetitive element (RLEP) region PCR amplification was carried out iPCR amplification volume that contained 3µl of template DNA, primers at a final concentration of 0.5M (forward and reverse). The RLEP primers used in this study were: Forward primer (F) 5′-TGC ATG TCA TGG CCT TGA GG-3′ and Reverse primer (R) 5′-CAC CGA TAC CAG CGG CAG AA-3′. The amplification was carried out in a thermal cycler under the of conditions of 95ºC for 5 min for initial denaturation followed by pre cycle under the condition of denaturation at 95ºC for 30s, annealing at 58ºC for 2 min, extension at 72ºC for 2 min, followed by cycle PCR for 45 cycles, each cycle consisting of denaturation at 95ºC for 30s, annealing at 58ºC for 2 min and extension at 72ºC for 2 min with a final extension at 72ºC for 10 min. PCR product containing amplified fragment of the target region was electrophoresed in a 2% agarose gel (Invitrogen) using Tris–Borate–EDTA buffer at 100 volts constant voltage.[15]

Result

63 water samples and 84 fomite swab samples from 29 Patients house and from various water bodies in and around patient's residence were collected [Table-1].In the present study, we analyzed 84 fomite samples of Bed, Towel and Door using LP-1 and LP-2 primers to amplify 129bp RLEP sequence, out of which 1 samples of door/floor and one Towel was found RLEP-PCR positive [Figure-1] in house of one MB case while rest were PCR negative. All water samples were found negative by PCR.

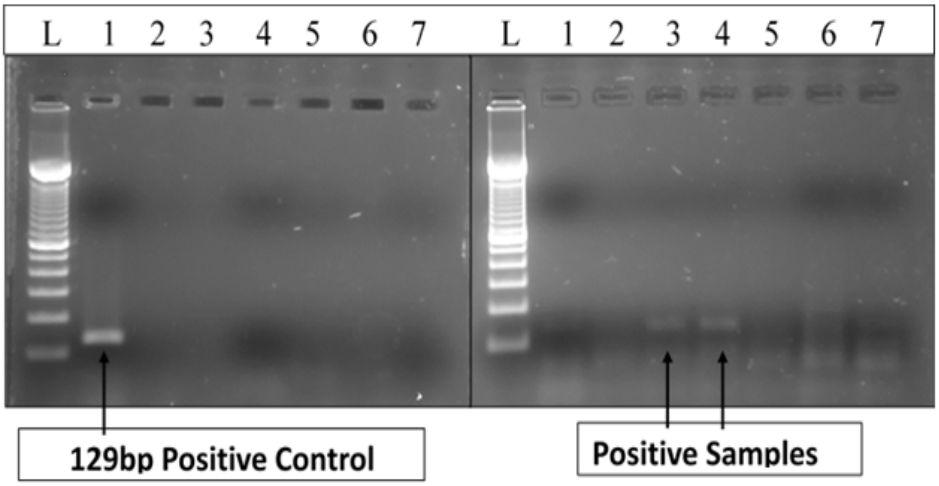

Figure 1: Gel-Doc result of amplified DNA, left side DNA ladder with positive control and right side shows positive samples

Table 1: List of number of samples collected according to sample type

S. No.	From	Number of samples
1	Bed	29
2	Towel	25
3	Door/floor	30
4	Drinking water	25
5	Drinage/ sewage water	13
6	Bathing water	14
7	Stagnant water (from storage body nearby house)	11
Total		147

Discussion

The latest update from the WHO titled "Global leprosy update, 2016: accelerating reduction of disease burden: states that – although there has been a significant reduction in prevalence of the disease worldwide since the mid-1980s to elimination levels, new cases continue to arise indicating continued transmission.[16]. This might be due to low level transmission of the disease in the community. Probable scientific reason for continuous transmission may be an infected person shedding Lepra bacilli in the surrounding environment which then continue to live in the environment by symbiotic or commensal mechanisms. [1]

In India, the National Leprosy Eradication Programme (NLEP) is the centrally sponsored health scheme of the Ministry of Health and Family Welfare, Government of India. Due to their efforts, from a prevalence rate of 57.8/10,000 in 1983, India has succeeded with the implementation of MDT in bringing the national prevalence down to "elimination as a public health problem" of less than 1/10,000 in December 2005 and even further down to 0.66/10,000 in 2016. In addition to achieving the national elimination target by the end of 2005, India by the end of March 2011–2012 succeeded in achieving elimination at the state level in 34 states/UTs out of the total of 36 states/UTs. Only the state of Chhattisgarh and the UT of Dadra & Nagar Haveli were yet to achieve elimination. [17]

Despite the above successes, the fact remains that India continues to account for 60% of new cases reported globally each year and is among the 22 "global priority countries" that contribute 95% of world numbers of leprosy warranting a sustained effort to bring the numbers down. Apart from the belief that an infected individual only is a source of infection many studies have found presence of *M. leprae* in various environmental settings.[1,10] As there are proof that the bacilli remains live for about 9 days outside human body even after drying [18] possibilities of environment as a source of infection could not be denied.

A study by Ploemacher et al states that the transmission of this disease is probably much more complicated than was thought before and involves several factors and pathways including the animal and environmental factors that might play a role in the persisting prevalence of leprosy.[19] Water bodies along with soil around the patient houses in high endemic area have been found having same and live organism [20].

Study by Lavania et al. (2006) showed *M. leprae* DNA was present in soil samples near Active Leprosy case house.[21] Turankar et al. (2012)also found the same result.[22] Turankar et al. (2016) repeated their earlier study and again found same result.[23]

Mohanty et al. (2016) found 25.4% of the soil samples and 24.2% of the water samples from the leprosy endemic area were positive for *M. leprae* 16S rRNA, while all the control samples which were from non endemic area were found negative.[24] Turankar et al. (2019) studied the association between non-tuberculous mycobacteria and *M. leprae* in the environment of leprosy endemic regions in India. They analysed soil (n = 388) and water (n = 250) samples for RLEP DNA and 16S rRNA. RLEP DNA was detected in 118 soil samples (30%) and 48 water samples (19%). 16S rRNA was detected in 53 soil samples (14%) and 30 water samples (12%)[25]

In this study we focused on fomite samples as they could be the direct source of infection and no similar study had been found during literature search. Pillow cover, handkerchiefs, towels, bed and door handles may get infected by various habits and practicesas they have direct contact with the nose which is probable most accepted entry and exit route of the infecting organism. Though we have found two swabs having *M.leprae* DNA by RLEP PCR this again adds to the fact that bacilli shedding may occur via nose and fomite may have a role in disease transmission. The RNA studies or animal inoculation studies would have more promising to findout the viability of the organism but were not done due to limitation of the study; but despite all effort when there is no further decline disease transmission rate may force us to think about environment as a source of *M. leprae* infection. Many of the studies done to findout transmission dynamics of *M. leprae* have been done years ago and using old microscopic/ animal inoculation techniques this study using PCR as a molecular diagnostic tool have increased sensitivity and specificity.

Conclusion

Our study highlights the presence of *Mycobacterium leprae* DNA on fomites such as pillow covers and towels, supporting the hypothesis that bacilli shedding via the nasal route occur and may contribute to environmental contamination and potential disease transmission. Although we did not assess viability through RNA analysis or animal inoculation, the detection of *M. leprae* DNA by sensitive PCR methods reinforces the possibility of fomites playing a role in ongoing transmission. Given the stagnation in reducing new cases despite elimination efforts, these findings suggest that environmental reservoirs, including contaminated fomites, should be considered in future strategies to interrupt leprosy transmission.

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