



Multiplex polymerase chain reaction of genetic markers for detection of potentially pathogenic environmental *Legionella pneumophila* isolates

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Background & objectives: Genomic constitution of the bacterium *Legionella pneumophila* plays an important role in providing them a pathogenic potential. Here, we report the standardization and application of multiplex polymerase chain reaction (PCR) for the detection of molecular markers of pathogenic potential in *L. pneumophila* in hospital environment.

Methods: Culture of the standard strains of *L. pneumophila* was performed in buffered charcoal-yeast extract agar with L-cysteine at pH 6.9. Primers were designed for multiplex PCR, and standardization for the detection of five markers annotated to *L. pneumophila* plasmid pLPP (11A2), lipopolysaccharide synthesis (19H4), CMP-N-acetylneuraminic acid synthetase (10B12), conjugative coupling factor (24B1) and hypothetical protein (8D6) was done. A total of 195 water samples and 200 swabs were collected from the hospital environment. The bacterium was isolated from the hospital environment by culture and confirmed by 16S rRNA gene PCR and restriction enzyme analysis. A total of 45 *L. pneumophila* isolates were studied using the standardized multiplex PCR.

Results: The PCR was sensitive to detect 0.1 ng/μl DNA and specific for the two standard strains used in the study. Of the 45 hospital isolates tested, 11 isolates had four markers, 12 isolates had three markers, 10 isolates had two markers, nine isolates had one marker and three isolates had none of the markers. None of the isolates had all the five markers.

Interpretation & conclusions: The findings of this study showed the presence of gene markers of pathogenic potential of the bacterium *L. pneumophila*. However, the genomic constitution of the environmental isolates should be correlated with clinical isolates to prove their pathogenic potential. Rapid diagnostic methods such as multiplex PCR reported here, for elucidating gene markers, could help in future epidemiological studies of bacterium *L. pneumophila*.

Key words Hospital infection control - *Legionella pneumophila* - multiplex polymerase chain reaction

The bacterium *Legionella pneumophila*, responsible for 90 per cent cases of Legionnaires' disease, is found universally in various natural and artificial aquatic environments, including hospital water distribution systems^{1,2}. Along with the

host-associated risk factors, the genetic constitution of *L. pneumophila* plays a crucial role in establishing an infection³⁻⁵. As Legionellosis is generally considered a preventable infection, identifying and treating the aquatic sources, especially in hospitals, could help in

the context of preventing hospital-acquired infection^{6,7}. In 2010, a group from the Netherlands reported five gene markers highly correlated with clinical strains of *L. pneumophila*, annotated to *L. pneumophila* plasmid pLPP (11A2), lipopolysaccharide synthesis (19H4), CMP-N-acetylneuraminic acid synthetase (10B12), conjugative coupling factor (24B1) and hypothetical protein (8D6)⁸. This study was undertaken with the objective of finding *L. pneumophila* in our hospital environment and to know the distribution of genes which are considered to have pathogenic potential. We report here the development of a multiplex polymerase chain reaction (PCR) for the detection of these five gene markers and its application in our hospital environmental isolates of *L. pneumophila*.

Material & Methods

This study was conducted in the department of Microbiology, All India Institute of Medical Sciences, New Delhi, India during 2012-2015. The study was approved by the Institutional Bio-Safety Committee (water and swab samples - 200 each approved for collection). No human subjects and/or animals were involved in this study.

Culture and DNA extraction: The standard strains ATCC 33152 *L. pneumophila* strain Philadelphia-1 and ATCC 33153 *L. pneumophila* strain Knoxville were grown on buffered charcoal-yeast extract agar (BD-BBL, France) supplemented with 4 per cent L-cysteine-HCl (HiMedia laboratories, Mumbai) in a candle jar incubated at 37°C for three days. Gram stain was done and blood agar was used as a negative control plate. DNA was extracted from the colonies by the boiling method. Briefly, colonies were boiled in sterile

distilled water for 10 min and centrifuged at 10,000 ×g to get the supernatant having the DNA.

PCR analysis and restriction enzyme analysis (REA): PCR detection of the 16S rRNA gene and REA were used for identification and confirmation of *L. pneumophila* standard strains and environmental isolates⁹. The restriction enzyme *TaaI* (HpyCH4III endonuclease, Thermo-Scientific Ltd., USA) was used for the differentiation of *L. pneumophila* and non-*pneumophila* *Legionella* species. Briefly, 15 µl of PCR product was digested with ×10 buffer Tango, 1U *TaaI* enzyme and adjusted with sterile water to final volume of 60 µl. The reaction was carried out in a water bath at 65°C for one hour and 20 µl of digested products were checked using 1.5 per cent agarose gel electrophoresis.

Multiplex PCR, sensitivity and specificity: Standardization of multiplex PCR for detection of five markers annotated to *L. pneumophila* plasmid pLPP (11A2), lipopolysaccharide synthesis (19H4), CMP-N-acetylneuraminic acid synthetase (10B12), conjugative coupling factor (24B1) and hypothetical protein (8D6) was done using the available gene sequences (Acc.nos. HM584933-HM584937). Primers (Table I) were selected and designed using NCBI Primer BLAST programme, and MPprimer design software¹⁰ was used for multiplex PCR primer design. The primers were synthesized commercially (Sigma, USA) and the DNA from both the standard strains were used for the multiplex PCR. Briefly, each reaction mix contained 5 µl of DNA, 5 µl of ×10 PCR buffer (Bangalore Genei, India), 1.5 µl of 10 mmol dNTP

Table I. Details of multiplex PCR primers designed for this study

Marker Identity	Forward/ Reverse Primer	Expected product size (bp)	Amplified product (bp)
11A2	5'-GGTGCATCGGAATGATAACCGCTCC-3' 5'-AGCGCGGACCTCTTTCTTGGGA-3'	355	~200/355
19H4	5'-ACGCCTCTTTGCTGAACCAGAG-3' 5'-CAGCACCAACATCCCGTGCA-3'	696	696
10B12	5'-GCTGGCGAGGTTGTTAGCGT-3' 5'-GCCAGGGTACTGAAGCCCCAT-3'	550	550
24B1	5'-AAAGCCGGTGGAGCTGGTTTCC-3' 5'-TTCGCCATGGGGTGTGTCGTTG-3'	236	236
8D6	5'-ATGCTGTTGAGGCTCATGCGGG-3' 5'-TCGCCTCTAACCGATGCAACCC-3'	102	102

mix (Thermo Scientific), 0.5 µl of 10 pmol/ µl each of five sets of primer, 0.5 µl of 3 U/µl Taq polymerase (Bangalore Genei) and sterile water adjusted to a final volume of 50 µl. The reaction was carried out in a thermal cycler (Applied Biosystems, USA) with the thermal programme having initial activation of 94°C for five minutes, 30 cycles of 94°C, 56°C, 72°C for 30 sec each and final extension of 72°C for 10 min. The primer pairs were checked individually for their sensitivity using various dilutions of standard strains' DNA and specificity with DNA isolated from various bacteria. The individual PCR products were purified using QIAquick Gel extraction kit (Qiagen, Germany) and sequenced commercially to confirm their identity.

Hospital environmental sample collection and processing: A total of 145 water samples (both potable drinking water and non-potable water used for bathing, washing, gardening, cooling tower water) and 200 swab samples (pre-water collection) were collected and processed from July 2012 to May 2014. The samples were collected from distal outlets and from AC cooling towers (basin beneath the tower). Temperatures of

the samples during the collection were also noted using a mercury thermometer (Zeal, England). The samples were treated with 0.1 N sodium thiosulphate and filtered with 0.22 µm mixed cellulose ester filters (Millipore, Germany). The filtrate was subjected to acid and thermal treatment and processed further for bacteriologic examination¹¹ (Fig. 1).

Multiplex PCR of environmental isolates: The multiplex PCR for five markers was applied as mentioned above to randomly chosen 45 hospital environmental isolates of *L. pneumophila* and one non-*pneumophila* *Legionella* species. Electrophoresis of the products was done in 1.5 per cent agarose gel stained with ethidium bromide, visualized under UV light and gel documentation system (Gel Doc EZ imager, Bio-Rad, USA). The presence or absence of the band was compared with the bands of standard strain ATCC 33153.

Results

Culture, PCR and REA confirmation: The culture of standard strains showed characteristic ground glass appearance. These were Gram-negative bacilli and no

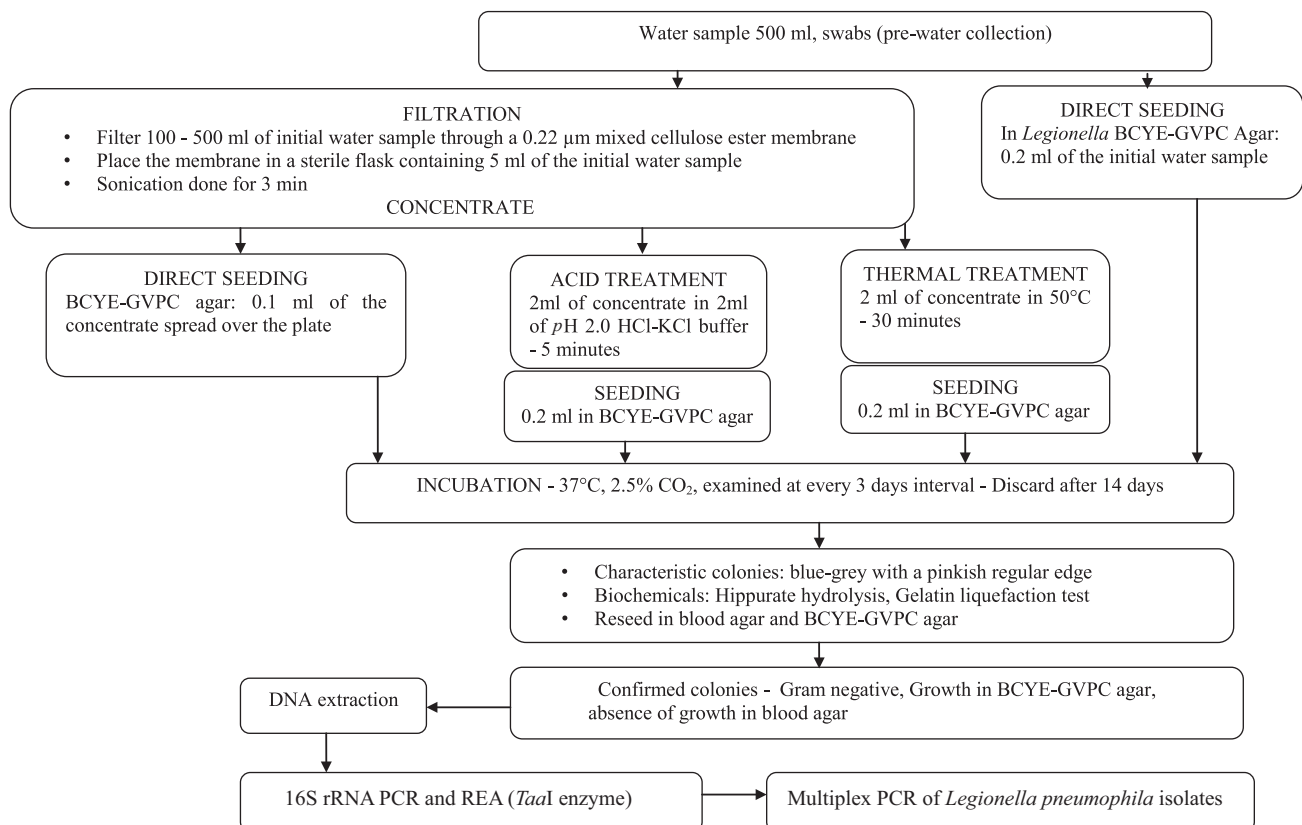


Fig. 1. Flowchart showing processing of water samples. BCYE-GVPC, buffered charcoal-yeast extract with Glycine, Vancomycin, Polymyxin B, and Cycloheximide; PCR, polymerase chain reaction; REA, restriction enzyme analysis.

growth was seen in blood agar. The 16S rRNA gene PCR showed 226 bp product which was digested into 180 bp and 46 bp by Taal restriction enzyme to confirm it as *L. pneumophila* (Fig. 2).

Multiplex PCR, sensitivity and specificity: The individual PCR for the markers showed expected product size, except for 11A2 marker. The PCR was found to be sensitive (Fig. 3A) to detect about 0.1 ng/ μ l of DNA and specific (Fig. 3B) for *L. pneumophila* standard strains used in the study. Both the ATCC standard strains of *L. pneumophila* used in the study showed a product at 200 bp region for marker 11A2. The ATCC strain 33153 showed the presence of all the five markers, whereas the marker 24B1 was not amplified in ATCC 33152. Sequencing of the gel-purified PCR products of ATCC strain 33153 showed 98-100 per cent identity with the GenBank sequences (<https://www.ncbi.nlm.nih.gov/nucleotide>) for the markers 19H4, 10B12, 24B1 and 8D6. The sequencing of marker 11A2 (pLPP) (~200 bp in our study) showed no significant similarity with *L. pneumophila* marker 11A2 available in the database (Gen bank Acc. no. HM584934). For marker 11A2, based on BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the available database, it was found that 95-98 per cent identity was seen with other *L. pneumophila* strains such as strain thunder bay, ATCC 43290, 2300/99 Alcoy, strain Corby, strain Philadelphia and strain Paris annotating to HeLa protein, cobalt/zinc/cadmium efflux RND transporter and HME protein importer family proteins.

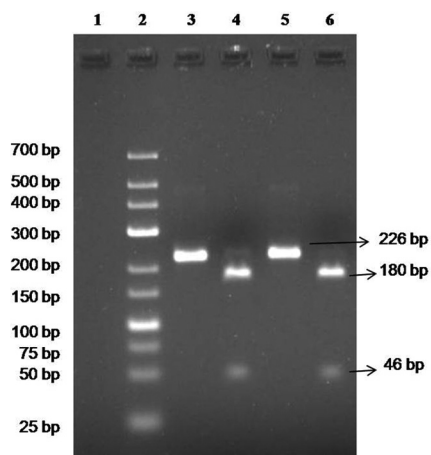


Fig. 2. 16S rRNA gene polymerase chain reaction (PCR) and restriction enzyme analysis (REA) of standard strains. Lane 1, negative control; lane 2, low range Marker (25-700 bp); lanes 3 & 4, PCR & REA of ATCC 33152 *Legionella pneumophila*, respectively; lanes 5 & 6, PCR & REA of ATCC 33153 *L. pneumophila*, respectively.

Culture, PCR-REA and multiplex PCR of environmental isolates: In a span of 22 months, the bacterium was isolated on 30 instances (21 instances by water samples only and nine instances by swabs samples). On 29 instances, *L. pneumophila* was isolated, and on one instance, non-*L. pneumophila* species was isolated as confirmed by PCR and REA. Isolation of the bacterium was seen in samples with temperature as low as 13°C to as high as 47°C. The bacterium was isolated in 21.5 and 6.3 per cent of non-potable and potable drinking water, respectively.

Of the 45 hospital environmental isolates of *L. pneumophila*, 11 isolates had four markers, 12 isolates had three markers, 10 isolates had two markers, nine isolates had one marker and three isolates had none of the markers. In our study, none of the isolates showed all the five markers in the multiplex PCR. All the isolates had the expected product size as compared to the bands in the standard strain ATCC 33153. However, of the 25 isolates, in the context of 11A2 marker, 19 showed the expected product size of 355 bp (Table II) in contrast to the standard strain which showed a band in 200 bp region (Fig. 4). Fig. 5A and B shows the representative picture of multiplex PCR in isolate numbers 27-45. The DNA of the one non-*pneumophila* *Legionella* species isolated from environment tested by the multiplex PCR showed one band at 355 bp which is the product size of 11A2 marker.

Discussion

For epidemiological purposes, sequence-based typing scheme of *Legionella* isolates involving seven genes is followed worldwide¹². Identification of genomic markers of pathogenic potential on *Legionella* bacterium is new.

The marker 24B1 (66.7%) was the most common in our isolates, followed by 11A2 (55.6%), 10B12 (48.9%), 8D6 (37.8%) and 19H4 (33.3%). The standard Philadelphia strain did not show the marker 24 B1. This means it is not necessary that the entire five markers be present together to give the strain a pathogenic potential. It is to be noted that the markers 24B1 and 11A2 are genes annotated to conjugative coupling factor and a plasmid, respectively, which may have a potential role in the horizontal gene transfer of the bacterium¹³. It is intriguing to note from our findings that in nine isolates where only one marker was found, seven isolates had 24B1 and two isolates showed 11A2 alone.

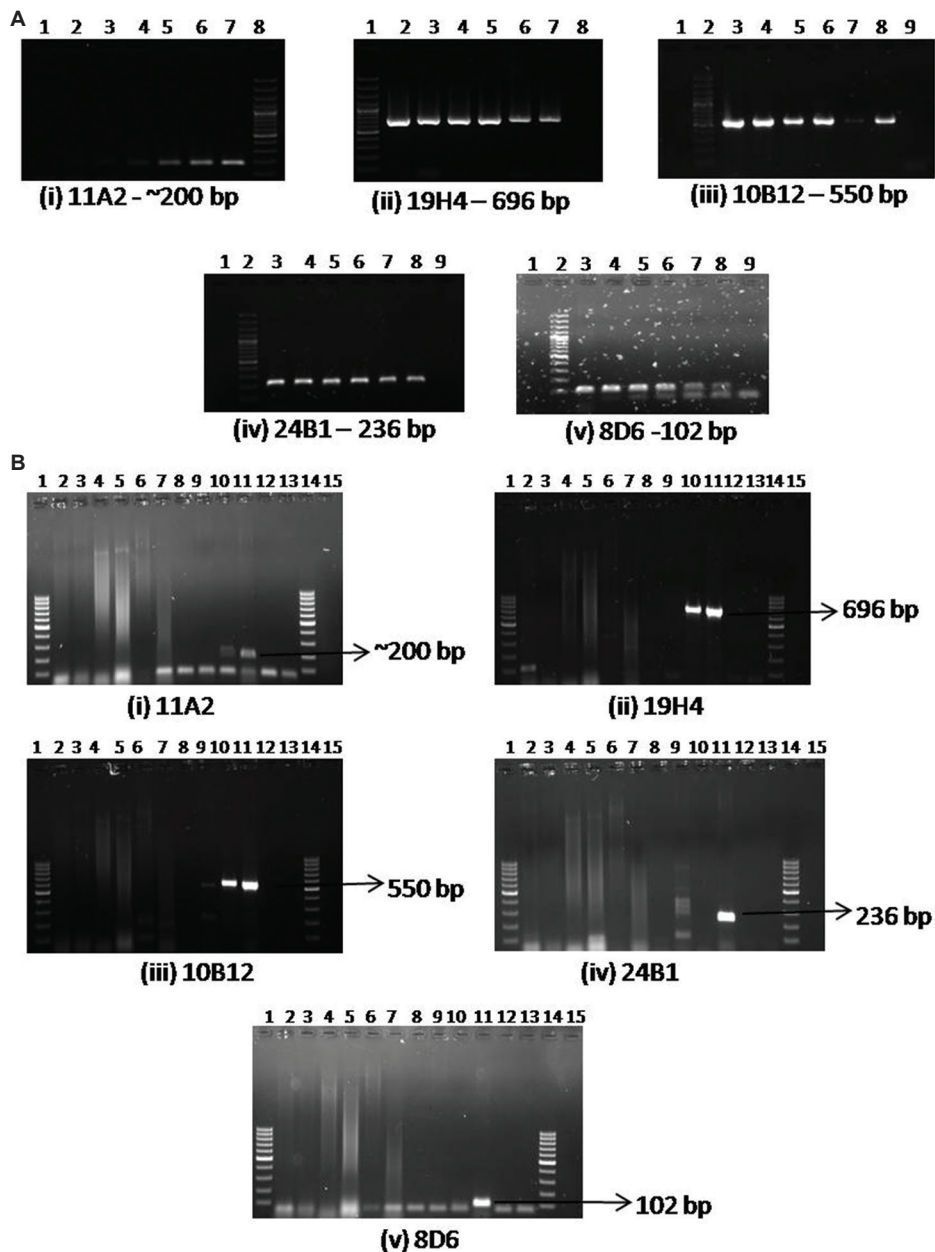


Fig. 3. (A) Sensitivity of PCR for individual markers: (i) 11A2 - Lane 1, negative control; lane 2, 1:1000 dilution (0.17 ng/ μ l); lane 3, 1:500 dilution; lane 4, 1:100 dilution; lane 5, 1:50 dilution; lane 6, 1:10 dilution; lane 7, ATCC 33153 DNA (177.4 ng/ μ l); lane 8, 100 bp ladder. (ii) 19H4 - Lane 1, 100 bp ladder; lane 2, ATCC 33153 DNA (177.4 ng/ μ l); lane 3, 1:10 dilution; lane 4, 1: 50 dilution; lane 5, 1:100 dilution; lane 6, 1:500 dilution, lane 7, 1:1000 dilution (0.17 ng/ μ l); lane 8, negative control. (iii) 10B12 - Lane 1, Blank; lane 2, 100 bp ladder; lane 3, ATCC 33153 DNA (177.4 ng/ μ l), lane 4, 1:10 dilution; lane 5, 1: 50 dilution; lane 6, 1:100 dilution; lane 7, 1:1000 dilution (0.17 ng/ μ l), lane 8, 1:500 dilution; lane 9, negative control. (iv) 24B1 - Lane 1, Blank, lane 2, 100 bp ladder; lane 3, ATCC 33153 DNA (177.4 ng/ μ l), lane 4, 1:10 dilution; lane 5, 1: 50 dilution; lane 6, 1:100 dilution; lane 7, 1:500 dilution; lane 8, 1:1000 dilution (0.17 ng/ μ l); lane 9, negative control. (v) 8D6 - Lane 1, Blank; lane 2, 100 bp ladder; lane 3, ATCC 33153 DNA (177.4 ng/ μ l); lane 4, 1:10 dilution; lane 5, 1: 50 dilution; lane 6, 1:100 dilution; lane 7, 1:500 dilution; lane 8, 1:1000 dilution (0.17 ng/ μ l); lane 9, negative control. **(B)** Specificity of PCR for individual markers: (i) 11A2, (ii) 19H4, (iii) 10B12, (iv) 24B1, (v) 8D6 - Lane 1, 100 bp ladder; lane 2, *Escherichia coli* DNA; lane 3, *Klebsiella pneumoniae* DNA; lane 4, *Proteus mirabilis* DNA; lane 5, *Pseudomonas sp.* DNA; lane 6, *Haemophilus influenzae* DNA; lane 7, *Neisseria meningitidis* DNA; lane 8, *Staphylococcus aureus* DNA; lane 9, *Mycobacterium tuberculosis* DNA; lane 10, *Legionella pneumophila* ATCC 33152 DNA; lane 11, *L. pneumophila* ATCC 33153 DNA; lane 12, negative control; lane 13, Blank; lane 14, 100 bp ladder.

All the markers showed expected product size with the standard strains used in the study except the marker

11A2. Of the 45 *L. pneumophila* isolates studied, 25 isolates showed the presence of 11A2 marker

Table II. Analysis of 45 hospital environmental isolates of *Legionella pneumophila* using multiplex PCR

Month/yr	Isolate	Marker identity	11A2	19H4	10B12	24B1	8D6	Total
Jul 2012	1	15S	+#	+	+	+	-	4
	2	16S	-	-	+	+	+	3
Aug 2012	3	9A	+#	-	-	-	-	1
	4	9B	+#	+	+	+	-	4
	5	9C	+	-	-	+	+	3
	6	11A	-	-	-	+	-	1
	7	11B	-	-	-	+	-	1
	8	15A	+#	-	-	+	+	3
	9	15B	+#	+	+	+	-	4
	10	15C	-	-	-	+	-	1
	11	16A	-	-	+	-	+	2
	12	20C	-	-	+	+	+	3
Jul 2013	13	2S	-	+	+	-	+	3
	14	4S	+#	+	+	-	+	4
	15	8S	+	+	+	+	-	4
	16	9S	+#	+	+	+	-	4
	17	11S	-	-	-	+	-	1
Aug 2013	18	16S	+	+	+	+	-	4
	19	8B	-	-	-	-	-	0
	20	8C	+#	+	+	-	-	3
	21	9B	+#	-	-	+	-	2
	22	9C	+	-	-	+	+	3
	23	11A	+#	+	+	-	-	3
	24	11B	+	-	-	+	-	2
	25	15C	+#	-	-	+	-	2
	26	19B	+#	-	-	-	-	1
	27	2B	+#	+	+	-	-	3
Oct 2013	28	5B	-	-	+	+	+	3
	29	11A	+#	-	+	-	-	2
	30	11B	-	-	-	-	-	0
Jan 2014	31	6B	+#	+	+	-	+	4
	32	6C	+#	+	+	-	+	4
	33	7A	-	-	-	-	-	0
	34	7C	+#	-	-	+	-	2
	35	12B	+#	+	+	-	-	3
	36	15B	-	-	-	+	-	1
	37	15C	-	-	-	+	+	2
May 2014	38	11B	-	-	-	+	+	2
	39	11C	-	-	-	+	-	1
	40	15A	-	-	-	+	+	2
	41	15B	-	-	-	+	-	1
	42	15C	-	-	-	+	+	2

Contd...

Month/yr	Isolate	Marker Identity	11A2	19H4	10B12	24B1	8D6	Total
	43	17B	-	-	+	+	+	3
	44	17C	+	-	+	+	+	4
	45	19B	+#	+	+	+	-	4
		Total +	25	15	22	30	17	
		%	55.6	33.3	48.9	66.7	37.8	

Isolate Identity: Numeral indicates the area number; letters indicate isolates from S- Swabs, A-Direct seeding post-filtration, B-Acid treatment, C- Thermal treatment. +, presence of amplified as compared to standard strain ATCC 33153 (four markers showed expected product size whereas marker 11A2 showed band at ~200 bp region in the standard strain); +#, presence of expected product size of 355 bp of 11A2 marker in the hospital environmental isolates; -, absence of products in multiplex PCR

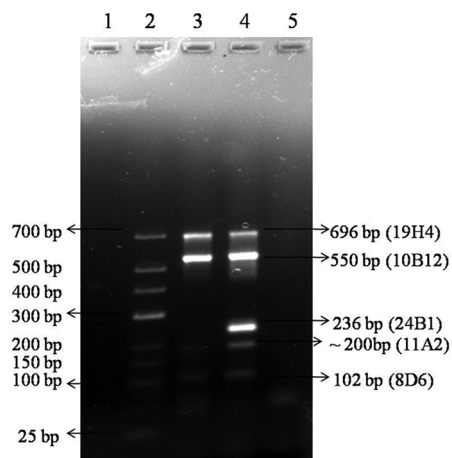


Fig. 4. Multiplex PCR of five markers in *Legionella pneumophila* standard strains ATCC 33152 and ATCC 33153 - Lane 1, Blank; lane 2, low range marker 25-700 bp size; lane 3, *Legionella pneumophila* ATCC 33152 DNA; lane 4, *L. pneumophila* ATCC 33153 DNA; lane 5, negative control.

(19 isolates showed an expected product size of 355 bp and six isolates showed a band in 200 bp region similar to the standard strain). This particular marker 11A2 is annotated to the plasmid of pLPP (Paris genome). The presence of an expected product for this plasmid in our isolates showed that this plasmid might not be specific to the Paris strain alone. Moreover, the presence of 11A2 marker in one non- *L. pneumophila* species isolated from our hospital showed that this plasmid was not specific for *L. pneumophila* but could be diversely present with other *Legionella* species also. Detailed studies for the presence of this plasmid as well as other markers in various *Legionella* species collected worldwide could give a better understanding of their ecological prevalence.

It is not necessary that the presence of these five markers in our isolates has proven their pathogenic potential because the ecological entities of continents

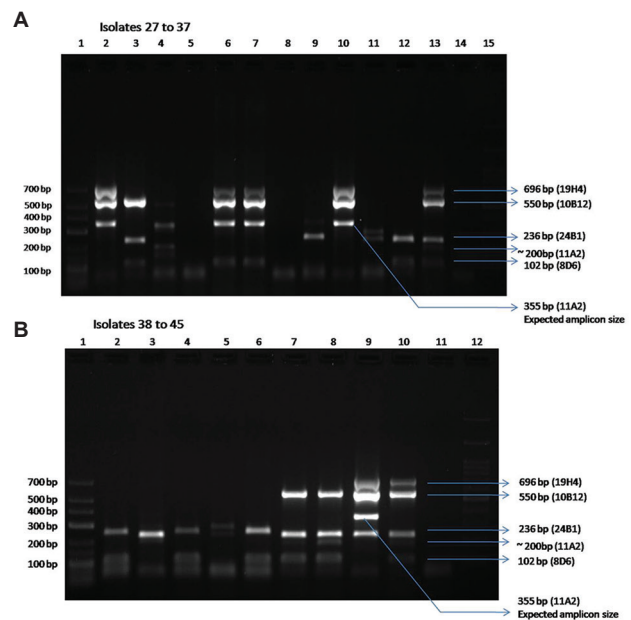


Fig. 5. Multiplex PCR of environmental *Legionella pneumophila* isolates (isolates 27 to 45 represented here). (A) Isolate 27 to 37 - Lane 1: low range Marker 25-700 bp, lane 2: isolate 27 (2B); lane 3, isolate 28 (5B); lane 4, isolate 29 (11A2); lane 5, isolate 30 (11B); lane 6, isolate 31 (6B); lane 7, isolate 32 (6C); lane 8, isolate 33 (7A); lane 9, isolate 34 (7C); lane 10, isolate 35 (12B); lane 11, isolate 36 (15B); lane 12, isolate 37 (15C); lane 13, positive control (ATCC 33153 *Legionella pneumophila*); lane 14, negative control; lane 15, 100 bp ladder. (B) Isolate 38 to 45 - Lane 1, low range Marker 25-700 bp; lane 2, isolate 38 (11B); lane 3, isolate 39 (11C), lane 4, isolate 40 (15A); lane 5, isolate 41 (15B); lane 6, isolate 42 (15C); lane 7, isolate 43 (17B); lane 8, isolate 44 (17C); lane 9, isolate 45 (19B); lane 10, positive control (ATCC 33153 *L. pneumophila*); lane 11, negative control; lane 12, 100 bp ladder.

are different. It is to be noted that the markers studied here may be applicable only to the European continent, especially in the Netherlands, for prediction, since genetic differences of the bacterium in different geographical areas have been documented¹⁴. Moreover, detailed mixed genome microarray studies have predicted different sets of markers in Dutch and French strains of *L. pneumophila*^{15,16}.

A repository of gene markers identified for specific geographical areas may help in epidemiological studies.

It is generally accepted that when 30 per cent or more of the water samples show *Legionella* growth, the system can be considered as contaminated¹⁷. In our study, the presence of *L. pneumophila* was observed in 15.4 per cent of the hospital water distribution systems. Apart from the distal outlets, the bacterium was isolated on more than three instances from two AC towers installed in the hospital. The cooling towers were cleaned and freshwater was infused and flushed out twice without chemical dosing. Water samples collected from these sites on April 2015 after cleaning turned out to be negative for the bacterium when tested by culture. However, in May 2015, cultures from the sites turned positive again showing that routine cleaning and monitoring is necessary to control the bacterium at least for tertiary care centres.

The first report of *Legionella* isolation in India showed culture positivity in nine per cent of clinical samples and 76 per cent of environmental samples¹⁸. Our earlier studies showed a seroprevalence of about 15 per cent (IgM antibodies) and antigenuria of about 17 per cent in community-acquired pneumonia cases^{19,20}. Only one fatal case associated with community-acquired *L. pneumophila* infection in a known immunocompromised individual has been reported²¹. A study group from south India has shown a culture positivity rate of about two per cent in clinical sample and about 33 per cent in environmental samples^{22,23}.

Our study had certain limitations. Only the bacteria which were recovered by culture were assessed for the presence of markers. However, viable but non-culturable forms²⁴ of this bacterium were not looked for and the specificity of the PCR with respect to other *Legionella* species was not checked. Moreover, it is essential to collect isolates from the patients and compare it with the environmental strains to get a clear picture of the infection-causing strains in India.

In conclusion, this study supports the idea that the distribution of genes of pathogenic potential in *L. pneumophila* may be diverse worldwide and rapid tests as the multiplex PCR reported here can help in epidemiological studies with respect to hospital infection control.

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Conflicts of Interest: None.

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