

# Genetic Diversity of the *Legionella pneumophila* dotA Gene Detected on Surfaces of Respiratory Therapy Equipment

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## Abstract

Legionellosis is a neglected disease due to the absence of well-defined clinical symptoms and difficulties in isolating the causal organism. *Legionella* spp. is known to colonize the lumen of respiratory therapy equipment(RTE) and evade conventional detection by entering the viable but non-culturable state. Monitoring these surfaces for *Legionella pneumophila* in addition to routine monitoring of water could aid in decreasing incidences of hospital-acquired infections by this pathogen. In this study swabs of different respiratory therapy equipment were tested for the presence of *Legionella* by conventional culture-based methods versus molecular detection of culture-independent template by polymerase chain reaction (PCR). Genetic diversity of the genes amplified were studied using bioinformatic tools. The dotA genes were genetically diverse indicating no clonality. This communication highlights that the persistence of virulence genes like dotA on abiotic surfaces can result in the mobilization of these genes to other species and give rise to virulent forms especially in a healthcare setting.

**Keywords:** Legionellosis, Nosocomial pneumonia, Respiratory therapy equipment, Tertiary care hospitals, Horizontal gene transfer, dot A

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## INTRODUCTION

*Legionella* is a Gram-negative aerobic bacterium which is a part of the natural aquatic environment<sup>1</sup>. The bacteria are ubiquitous in nature and are known for their ability to jump from the natural aquatic habitats into artificial water systems which can result in life threatening respiratory health risks<sup>2</sup>. An estimated 3 to 8% community acquired pneumonia are caused by *Legionella* spp and the greater risk of death due to this infection is common in immunocompromised and elderly patients<sup>3</sup>. The World Health Organization deems that the problem of *Legionella* is underestimated in developing countries due to the lack of awareness or failure in diagnosis. Legionnaire's disease (LD) is primarily spread via inhalation of contaminated aerosols. The sources of sporadic hospital-acquired infections caused by this pathogen are hospital showers and respiratory therapy equipment (RTE)<sup>4,5</sup>. Patients in hospitals are at greater risk of contracting respiratory infections when forced to inhale aerosols from respiratory devices that may contain Legionellae<sup>6,7</sup>. The water distribution system serves as a major source for *Legionella* but the mode of transmission continues to remain unclear<sup>8</sup>. Though *Legionella* comes under the purview of water quality, no guidelines are issued for the monitoring of *Legionella* on surfaces of respiratory therapy equipment where they can continue to reside even in the absence of water. This study was initiated to determine if *Legionella* can persist on respiratory therapy equipment in the absence of water.

## EXPERIMENTAL METHODOLOGY

### Sample collection

A total of 35 swab samples from respiratory therapy devices were collected aseptically using polyester sterile swabs. The sites for sampling were identified as follows: the compressor air outlet of nebulizers, the port of attaching mouthpiece of spirometer, the insertion tube surface of bronchoscope, and air inlet and outlet vents of ICU ventilators. Collected swabs were immediately immersed in sterile water and transported to the laboratory for analysis within 2 hours.

### Detection of *Legionella* by culture method

The isolation of *Legionella* was performed

as per US Centers for Disease Control and Prevention guidelines 2005. Buffered charcoal yeast extract (BCYE) agar containing 0.1% alpha-ketoglutarate and supplemented with glycine, vancomycin, polymyxin and cycloheximide (GVPC) was used for isolation of *Legionella pneumophila* and other related species. A volume of 0.1 ml of each sample suspension was plated on GVPC agar and incubated at 35°C with 2.5% carbon dioxide. The isolates that grew on GVPC were further enriched in BCYE broth which was used for DNA extraction by Cetyl trimethylammonium bromide (CTAB) method<sup>9</sup>. The quality and quantity of the extracted DNA was quantified using a UV nanodrop spectrophotometer (IMPLEN) and then used as templates for molecular detection.

### Molecular detection by polymerase chain reaction (PCR)

The isolated cultures were confirmed as members of genus *Legionella* by polymerase chain reaction (PCR) targeting genus specific 16S rRNA coding region using the JFP-JRP primer pair<sup>10</sup> and the species *L. pneumophila* by the presence of gene coding for the virulence factor *dotA*<sup>11</sup>. Alternatively, the swab suspension was directly used as template for culture-independent detection by PCR. All samples that were *dotA* positive by direct PCR detection were tested for amplification with eubacterial 16S rDNA universal primers<sup>12</sup>.

### Genetic relatedness of the *dotA* gene

The amplified PCR products of *dotA* were purified using QIAquick PCR purification kit and outsourced for Sanger sequencing (Eurofins Genomics India). A phylogenetic analysis of the *dotA* sequences was performed to compare the genetic relatedness of this gene among the reads obtained in this study to representative sequences from around the world available in NCBI nucleotide database. Sequences were aligned using Clustal Omega online tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and the phylogenetic tree was constructed using the Neighbor Joining algorithm available in MEGA X (version 10.0) with bootstrap tested for 100 replicates<sup>13</sup>.

## RESULTS

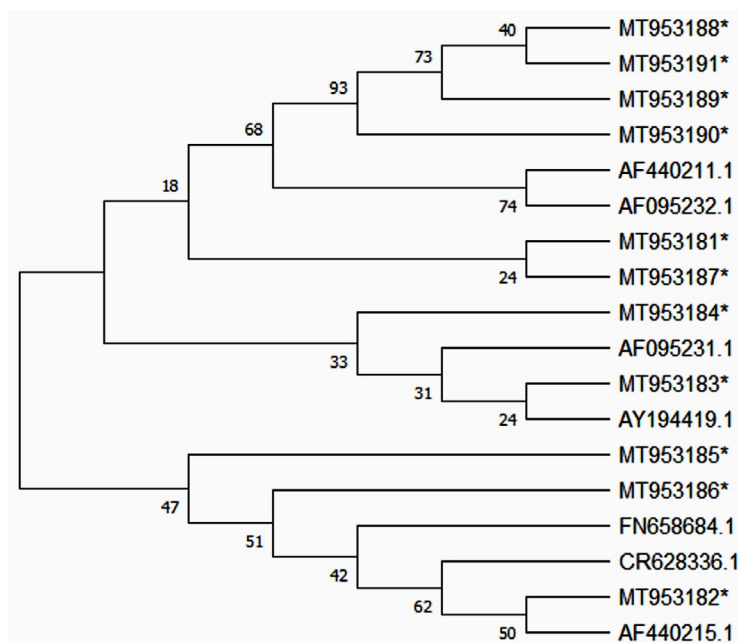
From a total of 35 swab samples, 23 isolates were obtained on GVPC agar. Of the 23 isolates that grew on GVPC only two isolates from

different nebulizer outlets were positive for the JFP-JRP region. None of the 23 isolates were positive for *dotA*. The two *Legionella* specific 16S rRNA sequences obtained in this study were deposited in GenBank and assigned the following accession numbers: MN784440 and MN795709. Sequence analysis of the JFP\_JRP amplicons showed >98% identity with *Legionella pneumophila* ATCC 33152 thus confirming the presence of *L. pneumophila* on dry surfaces of RTE.

Twelve of 35 swab samples were positive for the *dotA* gene when the swab suspensions (culture-independent) were directly used as templates for PCR. All amplicons were confirmed as the *dotA* gene by aligning the reads

obtained by Sanger sequencing with nucleotide reads in GenBank database using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The sequence reads were deposited in GenBank and assigned the accession numbers MT953181 to MT953191. The details of sequence used in construction of phylogenetic tree is provided (Supplementary data1). A phylogenetic tree for *dotA* region was constructed using the Neighbor-Joining method in MEGA X (Figure 1). The evolutionary distances were computed using the Maximum Composite Likelihood method. It could be inferred that there was no clonality among the *L. pneumophila* on the RTE surfaces even from the same healthcare facility.



**Fig. 1.** DotA Phylogenetic tree: *dotA* gene sequences obtained in this study (marked with \*) were aligned with sequences from various geographical locations retrieved from GenBank. The individual reads did not cluster together indicating that a diverse population of remnant *Legionella pneumophila* adhere onto respiratory equipment

**Table 1.** Isolation of *Legionella* by conventional method and detection by molecular methods

Sample type	Total no of Samples	Detected in culture			Direct PCR of sample		
		JFP	Dot A	Both	JFP	Dot A	Both
Nebulizers	15	2	0	0	1	5	1
Spirometers	4	0	0	0	0	2	0
Bronchoscopes	3	0	0	0	1	2	1
ICU ventilators (inlet & outlet)	13	0	0	0	0	3	0

Eubacterial 16S rDNA was amplified in all samples which were positive for *dotA*. However, eubacterial 16S rDNA amplicons from the swab samples yielded mixed reads.

## DISCUSSION

*Legionella* has emerged as major pathogen and may consequence to death. Knowledge of its affliction in India is limited to a few clinical studies<sup>14-17</sup> and fewer environmental studies<sup>15,18</sup>. There is a need to prioritize studies on this unexplored pathogen as the rates of ventilator associated pneumonia is fairly larger in India<sup>19,20</sup>. Though conventional culture technique is considered the gold standard for detection, use of molecular techniques like PCR have proven to increase the sensitivity for clinical diagnosis of *Legionella*<sup>21-24</sup>. Respiratory equipment like nebulizers and humidifiers have been implicated as sources of *Legionella* resulting in hospital-acquired Legionellosis. Surveillance of hospital water has been a primary prevention method of hospital-acquired legionellosis. *L. pneumophila* cell populations can potentially survive as free organisms for long periods by maintaining metabolic activity but temporarily losing cultivability<sup>25</sup>. Considering the fact that, *L. pneumophila* and other respiratory pathogens are able to enter into the viable but non culturable (VBNC) state easily, conventional culture techniques may no longer be the ideal method for detection. A lot of speculation over false positives being reported due to PCR have limited the applications of this technique.

Swabs from surfaces have yielded better recovery of *Legionella* than water samples<sup>14</sup>. A comparative account for the detection of *Legionella* by conventional and direct PCR method is our study depicted in table 1. Higher sensitivity for detection was observed in the direct PCR detection of genes from samples compared to culture technique which is in concordance to many previous reports<sup>26</sup>.

The JFP-JRP primer pair has been repeatedly reported to be specific for the genus *Legionella* and the same is proven based on sequence alignment (Supplementary Figure S1). Many studies have reported the concomitant occurrence of both JFP-JRP region and *dotA* in

clinical samples<sup>27</sup>. In our study, we observed many samples that were positive for *dotA* but not JFP-JRP region, hence the eubacterial 16S rDNA was amplified and attempted for sequencing. Mixed reads were obtained which indicated that these surfaces contain a mixture of bacteria. The non-specific growth observed in 23 of 35 samples on GVPC reiterates that these surfaces harbor many viable bacteria. The detection of *dotA* gene alone and absence of *L. pneumophila* 16S rRNA in some samples brings to light the need to elucidate the importance of this virulence gene in other potentially infectious organisms. The phylogenetic tree analysis of the *dotA* sequences revealed that this gene was not clonal and have been derived from many different strains. There was no geographical clonality among these sequences since though 4 of 11 (MT953188 to MT953191) clustered together they were not 100% identical. The other sequences were similar to *dotA* sequences reported from many other geographical locations of the world including Europe and America. The higher rate of detection of *dotA* compared to JFP-JRP region can be attributed to the fact that *dotA* detection was by a semi-nested PCR method.

Horizontal gene transfer has come to be of prime importance in the rise of new age pathogens. The Dot/Icm is the major virulence mechanism present in the pathogens like, *Helicobacter pylori*, *Bordetella pertussis*, *Brucella* sp, *L. pneumophila* and *Coxiella burnetii* utilize type IV secretion system for pathogenesis<sup>28</sup>. Of these, *L. pneumophila* and *Coxiella burnetii* are evolutionarily closely related organisms and both are human intracellular pathogens transmitted to humans through aerosols. Both organisms reside in alveolar macrophages inside an intact phagosome during infection which is mediated by the Dot/Icm.

Though in this study *Legionella* species have been detected only in two samples by the conventional culture technique, it is necessary to highlight that the presence of virulence genes like *dotA* on the therapy equipment surfaces may indicate the presence of VBNC forms of the pathogen *L.pneumophila*. Additionally, the persistence of the virulence gene on these surfaces can result in the mobilization of these genes to

other species and hence give rise to virulent forms. The *dotA* regions from the different samples were not identical indicating that these genes were from a variety of strains of *L. pneumophila* that are present on the various RTE within a healthcare setup.

RTEs are often used in individuals with compromised immunity and hence better cold sterilization techniques and routine monitoring using culture-independent techniques have to be made mandatory in hospitals.

The present study reveals the occurrence of *Legionella pneumophila* and associated species in respiratory equipment. To the best of our knowledge, this is the first report on the occurrence *Legionella* spp. in respiratory therapy equipment in tertiary care hospitals from this geographical location. This evidences that these RTE may serve as potential reservoirs of *Legionella* species and the usage of such respiratory equipment could transmit pathogenic *Legionella* spp in to the respiratory tract of patients.

Our observations support the need for concerted efforts to improve the proper maintenance of respiratory equipment as well as awareness among hospital personnel on the potential for aerosolizing pulmonary pathogens. Apart from routine monitoring of water systems for *Legionella*, we recommend monitoring swabs of respiratory therapy instruments for *Legionella* and other respiratory pathogens by culture-independent methods as an additional preventive measure against Legionellosis.

#### SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.15.2.20>

**Additional file:** Additional Table S1. Additional Figs. S1a and S1b.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

RV and JRMR did the experimentation and acquired funding. BKK and JRMR performed methodology. BKK verified the study. RV drafted the manuscript. BKK, IK and JRMR reviewed the manuscript. IK and JRMR did the final editing of the manuscript. All authors approved the manuscript for publication.

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#### ETHICS STATEMENT

Approval was obtained from the Institutional Ethics Committee (Ref INST.EC/EC/021/2015-16 dated 26.03.2015).

#### DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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