

## Prevalence and Virulence Factor Profiles of *Legionella pneumophila* Isolated from the Cases of Respiratory Tract Infections

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<http://dx.doi.org/10.22207/JPAM.11.1.10>

(Received: 03 January 2017; accepted: 20 February 2017)

*Legionella pneumophila* is one of the most severe causative agents responsible for lower respiratory tract infections. Pathogenesis of Legionella diseases is mainly occurred due to the activities of some latent virulence factors. The present investigation was carried out to study the prevalence rate of *L. pneumophila* and its putative virulence factors in the respiratory samples of patients. Three-hundred and fifty respiratory samples were taken from patients suffered from RTIs. All samples were cultured and their positive results were subjected to several PCR reaction. Prevalence in patients suffered from RTIs were 11.53%. *lidA* (70.58%), *rafF* (23.52%) and *lvhB* (17.64%) were the most commonly detected virulence factors in the *L. pneumophila* recovered from the respiratory samples. Considerable presence of virulent strains of *L. pneumophila* in patients showed that important public health issue facing hospitalized patients.

**Keywords:** *Legionella pneumophila*, Respiratory tract infections, Prevalence, Virulence factors.

Respiratory Tract Infections (RTIs) refers to any of a number of infectious diseases involving the respiratory tract. An infection of this type is normally further classified as an upper respiratory tract infection (URTI) or a lower respiratory tract infection (LRTI). Lower respiratory infections, such as pneumonia, tend to be far more serious conditions than upper respiratory infections, such as the common cold. These are one of the most common infectious diseases all-around the world with high rates of morbidity and mortality<sup>1,2</sup>.

One of the main pathogenic agent which are responsible for causing LTRI is Legionella species (spp.)<sup>2-5</sup>. Among all known species of Legionella, *Legionella pneumophila* (*L. pneumophila*) has the highest clinical impact and is a causative agent of various clinical complications like human legionellosis and

community-acquired and nosocomial pneumonia<sup>2-5</sup>. *L. pneumophila* is motile, gram-negative, fastidious and aerobic bacilli, non-fermentative, heterotrophic, catalase-positive, and urease and nitrate negative bacterium<sup>2-5</sup>. Confusion, headache, fever, abdominal pain, diarrhea, non-productive cough chills, and myalgia are the main clinical symptoms of RTIs and pneumonia caused by *L. pneumophila*<sup>2-5</sup>.

Pathogenesis of RTIs and also pneumonia caused by this bacterium are often derived from the presence of some putative virulence factors. In the other hand, to apprise the pathogenesis of diseases caused by *L. pneumophila*, study the latent virulence factors is required. Among all latent virulence factors which were attributed in pathogenesis of *L. pneumophila* infections, the poreformation protein *rtxA*, a number of effectors such as *rafF*, *lidA* and *lepA*, secretion system is prepilin peptidase *pilD* dependent and Legionella vir homolog (*lvh*) (a type IV secretion system involved in conjugation)<sup>6-8</sup>.

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The present investigation was carried out in order to study the prevalence rate and distribution of virulence factors in the *L. pneumophila* strains isolated from samples taken from Iranian patients suffered from RTIs.

## MATERIALS AND METHODS

### Samples collection

From January to November 2015, a total of 350 respiratory samples including Broncho Alveolar Lavages (BAL) (n=50) and also respiratory secretions (n=300) were sent to our laboratory center from hospitalized patients suffering from RTIs. In this study, a total of 150 respiratory samples were randomly selected and analyzed for presence of *L. pneumophila*.

### Bacterial isolation

Prior to culture, samples were centrifuged for 15 min at 2,500 rpm, and the top 7.5 ml of the resulting suspension was removed. The remaining cell concentrate was mixed and used for culture. Aliquots of 100 µL of prepared samples were spread on duplicate plates of aBCYE selective medium Agar (Difco Laboratories, Detroit, Mich., USA) and to plates containing L-cysteine (0.44mg mL<sup>-1</sup>), ferric pyrophosphate (0.250 mg mL<sup>-1</sup>), glycine (3.0 gL<sup>-1</sup>), vancomycin (0.0025 mg mL<sup>-1</sup>) and polymyxin B (0.006 mgmL<sup>-1</sup>), which are named aBCYE-GVP selective agar medium. Plates were incubated at 37°C in a humidified atmosphere without CO<sub>2</sub> during 5 days. Colonies with the typical ground glass appearance of Legionella were sub cultured on two nonselective media, sheep-blood agar and aBCYE agar without L-cysteine. Colonies that grew on aBCYEGVP but not on non-selective media were considered putative Legionella strains, and were Gram stained and subcultured on a selective medium. The identification of putative Legionella strains as *L. pneumophila* was carried out using Legionella specific latex reagents (Oxoid, Hampshire, England) and direct immunofluorescence assay with poly clonal rabbit sera (m-Tech Alpharetta, Ga., USA).

### PCR confirmation

*L. pneumophila* isolates were submitted to DNA extraction using the DNA extraction kit (Fermentas, Germany), according to the manufacturer's instructions. Set of primers for *lepA* gene of the *L. pneumophila* was designed by

Khedri *et al.* (2015) (9). The extracted DNA of each sample was kept frozen at -20°C until used. Primer sequences used for PCR, Legionella-F: 5'-GCTAATACCGCATAATGTCTGAGG-3' and Legionella-R: GGTGCTTCTGTGGGTAACG-3' (354 bp) were used for ramification of 16S ribosomal RNA (*16S rRNA*) gene of the *L. pneumophila*. PCR reactions were performed in a total volume of 25 µL, including 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH9.0), 0.1% Triton X-100, 200 µM dNTPs each (Fermentas, Germany), 25 pmol of each primer, 1.5 U of Taq DNA polymerase (Fermentas, Germany), and 3 µL (40-260 ng/µL) of DNA. The samples were placed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany) with an initial denaturation step at 95°C for 5 min, then amplified for 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and final extension step at 72°C for 5 min.

### Amplification of virulence factors

Table 1 represents list of primers, PCR conditions and volume of each reaction used from amplification of virulence factors Khedri *et al.* (2015) (9). All runs were done in a DNA thermocycler (Mastercycler gradient, Eppendorf, Germany). The PCR amplification products (10 µl) were subjected to electrophoresis in a 1% agarose gel in 1X TBE buffer at 80 V for 30 min, stained with ethidium bromide, and images were obtained in a UVIdoc gel documentation system (UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). A DNA of *L. pneumophila* ATCC 33152 was used as positive control and DNA of a laboratory isolate strain of *E. coli* as negative control.

### Statistical analysis

The data were analyzed using SPSS (Statistical Package for the Social Sciences) software and P values were calculated using Chi-square and Fisher's exact tests to identify statistically significant relationships for the distribution of *L. pneumophila* and virulence factors. A P value < 0.05 was considered statistically significant.

## RESULTS

A total of 150 samples were tested for presence of *L. pneumophila* and its putative virulence factors. Prevalence of *L. pneumophila*

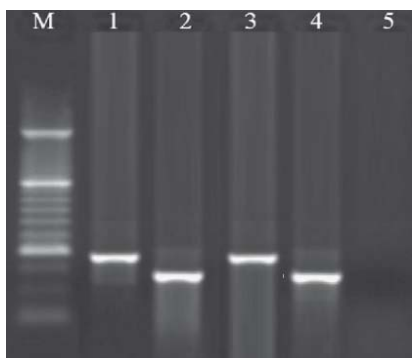
in patients of our study were 11.53%.

Figure 1-4 represent the results of the gel electrophoresis for putative virulence factors of the *L. pneumophila* recovered from samples of patients suffered from RTIs. Table 3 represents the distribution of putative virulence factors in the *L. pneumophila* strains isolated from the samples of

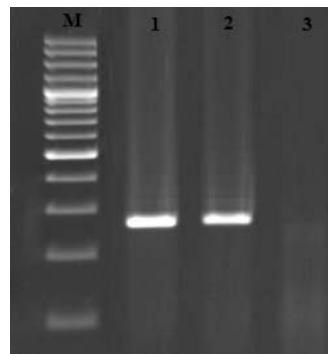
patients suffered from RTIs. All of the studied genes had the considerable prevalence in patients suffered from RTIs. We found that the most commonly detected virulence factors in the *L. pneumophila* recovered from the samples of studied samples were *lidA* (70.58%), *ralF* (23.52%) and *ivhB* (17.64%).

**Table 1.** List of primers and PCR conditions used for amplification of putative virulence factors of *L. pneumophila* strains isolated from patients suffered from RTIs (9)

Target gene	Primer sequence (5'-3')	Size of product (bp)	PCR program	Volume of reaction (50 µL)
<i>lidA</i>	F: CACAGGCTAAGGAAGACAGAGGC R: TAATTCCTTGACTTTTTCCGCAG	270	1 cycle: 94 °C - 6 min. 30 cycle: 95 °C - 30 s 64 °C - 1min 72 °C - 55 s 1 cycle: 72 °C - 7 min	5 µL PCR buffer 10X 2 mM Mgcl2 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template
<i>lepA</i>	F: GTTGGGCACTACAGTTATCTCTTC R: GTTAGTTACTACGGTTTCAATACGAC	354	1 cycle: 95 °C - 5 min. 30 cycle: 94 °C - 50 s 59 °C - 1min 72 °C - 1 min 1 cycle: 72 °C - 5 min	5 µL PCR buffer 10X 2 mM Mgcl2 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template
<i>ralF</i>	F: ACCAGCCCAGGATATGAACTTAC R: ATAGTAGCTTGTCGGATGTTTTG	230	1 cycle: 95 °C - 5 min. 30 cycle: 94 °C - 1 min 62°C - 1min 72 °C - 55 s 1 cycle: 72 °C - 10 min	5 µL PCR buffer 10X 2 mM Mgcl2 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template
<i>ivhB</i>	F: GTCAAACAACCTTCATTCAAACACC R: GGCAATAAATTCACAATCCAGAG	272	1 cycle: 95 °C - 5 min. 30 cycle: 94 °C - 1 min 60 °C - 1min 72 °C - 55 s 1 cycle: 72 °C - 5 min	5 µL PCR buffer 10X 2 mM Mgcl2 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template
<i>rtxA</i>	F: ATTGCTTTTTTCAGGTATCACTAACG R: ATTCGTTGATGTACTAATAGGCTGG	265	1 cycle: 95 °C - 5 min. 30 cycle: 94 °C - 1 min 62 °C - 1min 72 °C - 1 min 1 cycle: 72 °C - 6 min	5 µL PCR buffer 10X 2 mM Mgcl2 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template



**Fig. 1.** Results of the gel electrophoresis for identification of *ralF* and *lepA* genes of the *L. pneumophila* strains. M: 100 bp DNA ladder (Fermentas, Germany), 1: Positive sample for *lepA* gene (354 bp). 2: Positive sample for *ralF* gene (230 bp), 3 and 4: Positive Control



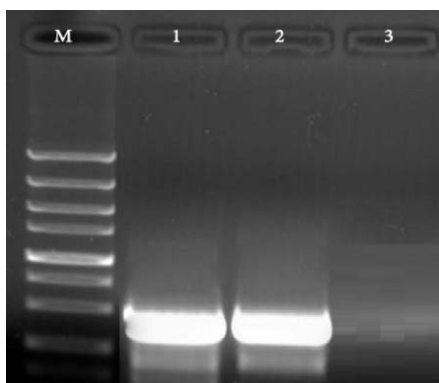
**Fig. 2.** Results of the gel electrophoresis for identification of *lvhB* gene of *L. pneumophila* respiratory samples, M:100 bp DNA ladder (Fermentas, Germany), 1: Positive control, 2: Positive samples for *lvhB* gene (272 bp) and 3: Negative control

**Table 2.** Total prevalence of *Legionella pneumophila* in the respiratory samples taken from patients suffered from RTIs.

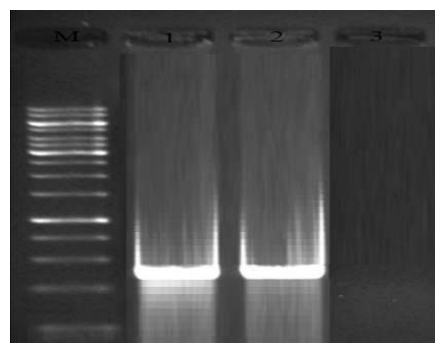
Types of samples	No. samples collected	Prevalence of <i>L. pneumophila</i> (%)	PCR confirmation (%)
Patients	150	17 (11.33)	17 (11.33)

**Table 3.** Distribution of putative virulence factors in the *Legionella pneumophila* strains isolated from the respiratory samples of patients suffered from RTIs

Types of samples (No. positive)	Distribution of putative virulence factors (%)				
	<i>lidA</i>	<i>lepA</i>	<i>ralF</i>	<i>lvhB</i>	<i>rtxA</i>
Patients (17)	12 (70.58)	2 (11.76)	4 (23.52)	3 (17.64)	1 (5.88)



**Fig. 3.** Results of the gel electrophoresis for identification of *lidA* gene of *L. pneumophila* in respiratory samples, M:100 bp DNA ladder (Fermentas, Germany), 1: Positive control, 2: Positive samples for *lidA* gene (270 bp band) and 3: Negative control



**Fig. 4.** Results of the gel electrophoresis for identification of *rtxA* gene of *L. pneumophila* in respiratory samples, M:100 bp DNA ladder (Fermentas, Germany), 1: Positive control, 2: Positive samples for *rtxA* gene (265 bp band) and 3: Negative control

## DISCUSSION

This work was carried out to study the prevalence of *L. pneumophila* in the samples taken from patients suffered from RTIs. As far as we know, the present investigation is the first prevalence report of the *L. pneumophila* in samples taken from patients in Iran. As it showed, the prevalence of *L. pneumophila* in patients were 11.53%. Faradonbeh *et al.* (2015) (10) revealed that sex and age of patients and history of smoking were the main risk factors for occurrence of RTIs caused by *L. pneumophila*. They showed that the prevalence of *L. pneumophila* among samples was 12% which was higher than our results. Among all previous investigations which were conducted on the prevalence of *L. pneumophila* in clinical samples<sup>11-14</sup>, Ghotaslou *et al.* (2013)<sup>11</sup>, Yu *et al.* (2008) (12) and Azara *et al.* (2006)<sup>13</sup> reported that higher than 20% of samples were positive for *L. pneumophila*, while Chaudhry *et al.* (2000)<sup>14</sup> reported lower prevalence. Similar findings have been reported by Faradonbeh *et al.* (2015) (Iran)<sup>10</sup>, Ngeow *et al.* (2005) (Malaysia, Thailand, China, Philippines, Taiwan, South Korea, Singapore and Indonesia)<sup>15</sup>, Nagalingam *et al.* (2005) (Trinidad and Tobago)<sup>16</sup> and Amemura-Maekawa *et al.* (2010) (Japan)<sup>17</sup>.

The second part of our investigation focused on the detection of putative virulence factors in the *L. pneumophila* strains of patients. As it showed, the most commonly detected virulence factors were *lidA*, *ralF* and *lvhB*. Similar findings were reported by Khedri *et al.* (2015)<sup>9</sup>. They showed that the prevalence of *L. pneumophila* was 12%. Total distribution of *lepA*, *lidA*, *ralF*, *rtxA* and *lvhB* virulence factors in the *L. pneumophila* strains of patients suffered from RTIs were 11.11%, 50%, 27.77%, 5.55% and 16.66%, respectively. Unfortunately, the numbers of investigations which were conducted on the distribution of putative virulence factors in *L. pneumophila* strains of clinical samples were low. In a study which was conducted on Australia by Huang *et al.* (2006)<sup>18</sup>, prevalence of *rtxA* and *lvh* genes in patients suffered from RTIs caused by *L. pneumophila* were 64.4% and 57.6%, respectively. High prevalence of *lidA*, *ralF* and *lepA* genes was also described previously by Newton *et al.* (2006)<sup>19</sup>, Gilmour *et al.* (2007)<sup>20</sup>, Huang *et al.* (2006)<sup>18</sup> and Khedri *et al.* (2015)<sup>9</sup>.

*RtxA* positive strains of *L. pneumophila* have a high ability to enter to monocytes and epithelial cells, and increased cytotoxicity and intracellular duplication<sup>21-23</sup>. Presence of *lepA*, *lidA*, *ralF*, *rtxA* and *lvhB* virulence genes is essential for the survival and growth of *L. pneumophila* in macrophages, for avoidance of phagosome acidification and lysosome fusion and is necessary for instruction of apoptosis in human macrophages<sup>21-23</sup>.

## CONCLUSIONS

In conclusion, we identified a large number of virulent strains of *L. pneumophila* in the samples of sick persons. Higher prevalence of bacteria in patients suffered from RTIs with respect to the high distribution of *lepA*, *lidA*, *ralF*, *rtxA* and *lvhB* virulence genes are two important findings of our study. Further studies need to be done to determine the exact roles of various virulence factors of *L. pneumophila*.

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