RESEARCH ARTICLE

Correlation between *Legionella pneumophila* serogroups isolated from patients with ventilator-associated pneumonia and water resources: a study of four hospitals in Tehran, Iran

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Abstract

Legionella pneumophila (*L. pneumophila*) is one of the main pathogens, causing pneumonia and respiratory tract infections, especially in patients with ventilator-associated pneumonia (VAP). This study aimed to approve the hypothesis that the serogroup distribution of *L. pneumophila* isolates from patients is correlated with *Legionella* strains in the environment. A total of 280 bronchoalveolar lavage (BAL) samples from VAP patients admitted to the intensive care unit (ICU) as well as 116 water samples from different sources in four hospitals in Tehran, Iran, were evaluated for the presence of *L. pneumophila* infection by culture, nested polymerase chain reaction (PCR), real-time PCR, and sequencing for genetic diversity. The molecular and culture methods found 24 (8.6%) and 5 (1.8%) samples to be positive for *L. pneumophila* in VAP patients, while they found 23 (19.8%) and 8 (6.9%) positive samples in water resources, respectively. The sequencing results indicated that all positive clinical samples and 14 (60.8%) environmental samples were belonged to *L. pneumophila* serogroup 1. Smoking, age, length of ICU stay, and duration of ventilator use had strong relationship with *L. pneumophila* infectivity. In conclusion, this is the first report from Iran to determine minor differences in the serogroup distribution of environmental and clinical strains. However, further studies are needed to confirm this relationship in different regions of Iran.

Keywords *Legionella pneumophila* · Ventilator-associated pneumonia · Serogroup 1 · Water resources

Abbreviations

L. pneumophila	Legionella pneumophila	PCR	Polymerase chain reaction
VAP	Ventilator-associated pneumonia	BCYE	Buffered charcoal yeast extract
BAL	Bronchoalveolar lavage	DST	Drug susceptibility test
ICU	Intensive care unit	*Mip*	*M*Acrophage infectivity potentiator
		DFA	Direct fluorescent antibody

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Introduction

Ventilator-associated pneumonia (VAP) is the most common intensive care unit (ICU)-acquired infection and a leading cause of mortality in patients admitted to ICUs (Al-Omari et al. 2021).

Bacterial agents related to VAP vary depending on several factors, such as duration of mechanical ventilation, local ecology, timing and cumulative exposure to antimicrobials, hospitalization, ICU stays before VAPs, and occurrence of any potential epidemic phenomena in the ICU setting. Common gram-negative bacteria associated with VAP include *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter species,* and *Escherichia coli; Staphylococcus aureus,*

and the main gram-positive bacterium are *Staphylococcus aureus*. Other bacteria, such as *Mycoplasma pneumoniae* and *Legionella pneumophila* (*L. pneumophila*), have been also detected (Papazian et al. 2020; Torres et al. 2021).

Several studies have indicated that 2–15% and above 30% of community- and hospital-acquired pneumonia infections are caused by *L. pneumophila*, respectively (Chahin & Opal 2017; Torres et al. 2021). However, *L. pneumophila* cannot be distinguished from other pneumonia infections according to the clinical signs. Overall, early diagnosis and treatment of infection can be helpful in reducing the mortality rate (Khosroshahi et al. 2015).

The severity of legionellosis varies from a mild febrile disease to a severe and even fatal form of pneumonia, caused by exposure to *L. pneumophila* in water. The most common route of *L. pneumophila* transmission is inhalation of contaminated aerosols. Aerosol sources correlated with *Legionella* transmission include hot- and cold-water systems, humidifiers, whirlpool spas, and air conditioning cooling towers. These sources are known to involve the respiratory system. Individuals, especially susceptible hospitalized patients, can be also infected with contaminated water or aspiration of ice water (Gonçalves et al. 2021; Torres et al. 2021).

Although the incidence of legionellosis has been reported worldwide, its exact occurrence remains undetermined due to inappropriate monitoring of infected cases. Early diagnosis of this disease depends on the physician's increased awareness of the disease and availability of the required equipment for detection (Yaslianifard et al. 2019).

The use of molecular epidemiological techniques to characterize clinical and environmental isolates can provide data for specific geographical areas and allow phylogenetic and population analyses. Overall, identification of these characteristics is essential for a better understanding of the transmission routes of bacteria in the environment. The sequence type distribution of *L. pneumophila* in North America, Europe, and Asia revealed the predominance of specific sequence types in the clinical isolates and indicated that most clinical cases and outbreaks were caused by a decrease in the number of *L. pneumophila* isolates (Bianchi et al. 2016; Quero et al. 2018).

With this background in mind, the current study aimed to evaluate and compare clinical and environmental *L. pneumophila* isolates from water resources in four hospitals of Tehran, Iran.

Methods

Sample collection from VAP patients and culture

In this study, 280 bronchoalveolar lavage (BAL) samples were collected from intubated patients, admitted to the ICUs of four teaching hospitals affiliated to Tehran University of Medical Sciences, Tehran, Iran. The fresh samples were divided into two parts; one part was used for cultivation, and the other part was used for the polymerase chain reaction (PCR) assay (stored at -20 °C until testing).

Before culture, sample decontamination was required to decrease the contaminating microbiota in respiratory samples by diluting (1:10) the BAL samples in a low-pH KCl/HCl buffer (pH 2.2) and incubating them at room temperature for four minutes before plating the suspension onto a buffered charcoal yeast extract (BCYE) agar medium (Sigma Aldrich, France). The plates were incubated at 37 °C in a 5% CO_2 atmosphere with humidity and kept for one week (Edelstein and Lück 2015). Also, *L. pneumophila* ATCC33152 was used as a positive control. Bacterial colonies emerged following the fifth day.

Sample collection from water resources and cultures

In the present study, 116 water samples from ICUs, where VAP patients were hospitalized; were collected from different water sources, such as water tap, air conditioners, and drinking water coolers in sterile, non-toxic 1000-mL plastic bottles; and then transferred to the laboratory in less than 4 h.

The water samples were centrifuged at 3000 g for 30 min (25 °C). Next, 500 µL of precipitation was treated with 4.5 mL of acid wash buffer, as mentioned above. After shaking, 200 µL of suspension was inoculated on the BCYE agar, supplemented with glycine, vancomycin, cyclohexamide, and polymyxin B (Edelstein and Lück 2015).

The drug susceptibility test (DST) for antimicrobial agents was also performed using the disk diffusion method for all positive culture isolates from clinical and environmental samples (Fard et al. 2012, Rahimi and Vesal 2017).

Nested PCR and real-time PCR methods

Purified genomic DNA was extracted from BAL samples, water samples, and bacterial colonies using a PROBA-NK DNA Extraction Kit DNA-Technology Company, Moscow, Russia), according to the manufacturer's instructions.

In this study, we identified *Legionella* species by both nested and real-time PCR assays, using the macrophage infectivity potentiator (*mip*) gene primer, as previously described (Fiume et al. 2005; Wilson et al. 2003). The selection of *mip* gene for screening was based on its discriminative power and frequent use in other studies (Ahmadrajabi et al. 2016).

DNA sequencing and phylogenetic analysis

For *L. pneumophila* genotyping, the *mip* gene from the isolates was amplified using primers, Legmip_f and Legmip_r, as previously described (Ratcliff et al. 1998). First, a High-Pure PCR Product Purification Kit (Sigma-Aldrich, Missouri, USA) was used for the purification of PCR products. Next, an automated ABI sequencer (Applied Biosystems, Foster City, CA, USA) was applied for PCR sequencing. Genotyping was performed using a phylogenetic analysis, with reference sequences selected from the GenBank database. Moreover, a phylogenetic tree reconstruction was performed in MEGA version 6.0 software (http://www.megas oftware.net), using the neighbor joining method and K2P model with 500 bootstrap replicates.

Statistical analysis

SPSS for Windows Version 22.0 (2016; IBM Corp., Armonk, NY, USA) was applied for data analysis. The normal distribution of continuous variables was determined by Shapiro–Wilk test. Pearson's Chi-square and Mann–Whitney U tests were also performed to evaluate quantitative and continuous variables, respectively. Besides, for evaluating the relationships between the risk factors and *L. pneumophila* infectivity, a multivariate logistic regression analysis was carried out using the Hosmer–Lemeshow test. Two-tailed P value > 0.05 was considered statistically significant.

Results

Detection of L. pneumophila in VAP patients

Of 280 BAL samples, 24 (8.6%) were positive in nested and real-time PCR. Of 24 PCR-positive samples, five (20.8%) showed growth in the BCYE agar. These patients (A1, A2, B1, C1, and D1) were infected with a large number of bacteria in the culture. The direct fluorescent antibody (DFA) test revealed that these isolates belonged to the *L. pneumophila* serogroup 1.

The mean age of positive cases was 69.5 ± 20.3 years, and the male-to-female ratio was 2.0 (16 vs. 8). The mean duration of ventilator use and ICU stay were 26.5 ± 7.6 and 33.0 ± 7.8 days, respectively (Table 1). Also, among 24 positive cases, 18 (75%) were smokers.

All positive samples were examined to determine the most common viral, fungal, and bacterial infectious agents of the respiratory tract, such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Candida albicans, Aspergillus* spp*., Mucor* spp., influenza A and B viruses, coronaviruses, and rhinoviruses. All positive samples were negative for these agents.

The DST revealed that all five isolates were susceptible to rifampicin (10 μg), levofloxacin (5 μg), tetracycline (15 μg), clarithromycin (15 μg), and erythromycin (15 μg) and resistance to streptomycin (10 μg), kanamycin (30 μg), and doxycycline (30 μg).

Table 1 Comparison demographics parameters between patients with and without *Legionella pneumophila* infection based on molecular methods

Variables	Positive ($n = 24$)	Negative ($n = 256$)	P value
Mean age $\pm$ SD	69.5 ± 20.3	60.2 ± 16.3	0.010*
Gender (male/female)	16/8 (66.7/33.3%)	162/94 (63.3/36.7%)	0.742
Hospitals			0.692
A	8 (11.4%)	62 (88.6%)	
B	6 (8.6%)	64 (91.4%)	
C	4 (5.7%)	66 (94.3%)	
D	6 (8.6%)	64 (91.4%)	
Length of ICU stay (day $\pm$ SD)	33.0 ± 7.8	22.9 ± 11.3	< 0.001*
Duration of ventilator use (day $\pm$ SD)	26.5 ± 7.6	17.7 ± 9.3	< 0.001*
Underlying disease			0.513
Respiratory disease	6 (13.9%)	37 (86.1%)	
Diabetes	6 (8.7%)	63 (91.3%)	
Surgical (kidney, lung, colon, etc.)	6 (10.3%)	52 (89.7%)	
Internal disease	3 (6.4%)	44 (93.6%)	
Heart disease	3 (4.8%)	60 (95.2%)	
Smoking	18 (75.0%)	95 (37.1%)	0.025*

SD Standard deviation; *statistically significant (< 0.05)

Detection of L. pneumophila in water resources

Out of 116 water samples, 23 (19.8%) and 8 (6.9%) were positive for *L. pneumophila* based on PCR and culture methods, respectively; this indicates the higher sensitivity of molecular methods compared to the culture method. The *L. pneumophila* distribution in water resources is shown in Table 2. In this study, the most common contaminated sources in the hospitals included water in ICUs and tap water. All *L. pneumophila* isolates from cultures belonged to serogroup 1, as determined by the DFA test.

The DST revealed that all eight isolates were susceptible to rifampicin (10 μg), levofloxacin (5 μg), and erythromycin (15 μg).

Phylogenetic analysis of L. pneumophila by genotyping the mip gene

To confirm genotyping by the DFA test, the PCR product of the *mip* gene was sequenced as previously described (Ratcliff et al. 1998). All positive samples based on nested and real-time PCR were amplified and sequenced to construct a phylogenetic tree. The sequencing results of the clinical samples revealed that all positive samples belonged to serogroup 1, while in environmental samples, only 14 (60.8%) belonged to serogroup 1; other samples belonged to serogroups 2, 3, 8, and 10. The sequences of *L. pneumophila* indicated more

than 98% homology to serogroup 1 sequences available in the GenBank database (Fig. 1).

Factors related to L. pneumophila infectivity

We performed a multivariate logistic regression to assess the correlations between the clinical parameters and *L. pneumophila* infectivity. The factors independently related to *L. pneumophila* infectivity include age (OR, 1.164; 95% CI, 1.009–3.089; $P = 0.010$), smoking (OR, 1.895; 95% CI, 1.001–1.995; $P = 0.025$), length of ICU stay (OR, 0.951; 95% CI, 0.885–0.998; $P < 0.001$), and duration of ventilator use (OR, 0.921; 95% CI, 0.863–0.984; $P < 0.001$) (Table 3).

Discussion

In this preliminary study, we investigated and compared the clinical and environmental *L. pneumophila* serogroup isolates in four hospitals. According to our findings, the serogroup distribution of *L. pneumophila* isolates from patients was almost similar to the distribution of *L. pneumophila* isolates from the environment. Previous reports from different regions have suggested a possible similarity between these distribution patterns (Kozak-Muiznieks et al. 2014; Quero et al. 2018), although they have not been performed

Table 2 Detection of *Legionella pneumophila* by *mip* gene and culture in water resources collected from different cooling water systems in four hospitals in Tehran, Iran

Hospital	Sources	Total no. of samples	Nested PCR	Real-time PCR	Culture
A	Intensive care	4	2 (50.0%)	2 (50.0%)	1 (25.0%)
	Operating room	5	-	-	-
	Tap water	9	5 (55.6%)	5 (55.6%)	1 (11.1%)
	Air conditioner	5	2 (40.0%)	2 (40.0%)	1 (20.0%)
	Hospital chiller	6	3 (50.0%)	3 (50.0%)	1 (16.7%)
B	Intensive care	4	1 (25.0%)	1 (25.0%)	-
	Operating room	5	1 (20.0%)	1 (20.0%)	-
	Tap water	9	2 (22.2%)	2 (22.2%)	1 (11.1%)
	Air conditioner	5	1 (20.0%)	1 (20.0%)	-
	Hospital chiller	6	1 (16.7%)	1 (16.7%)	-
C	Intensive care	4	1 (25.0%)	1 (25.0%)	1 (25.0%)
	Operating room	5	-	-	-
	Tap water	9	1 (11.1%)	1 (11.1%)	-
	Air conditioner	5	-	-	-
	Hospital chiller	6	-	-	-
D	Intensive care	4	1 (25.0%)	1 (25.0%)	1 (25.0%)
	Operating room	5	-	-	-
	Tap water	9	1 (11.1%)	1 (11.1%)	-
	Air conditioner	5	-	-	-
	Hospital chiller	6	1 (16.7%)	1 (16.7%)	1 (16.7%)
Total		116	23 (19.8%)	23 (19.8%)	8 (6.9%)

Fig. 1 The phylogenetic trees constructed by the *mip* gene sequences in different *Legionella pneumophila* serogroups
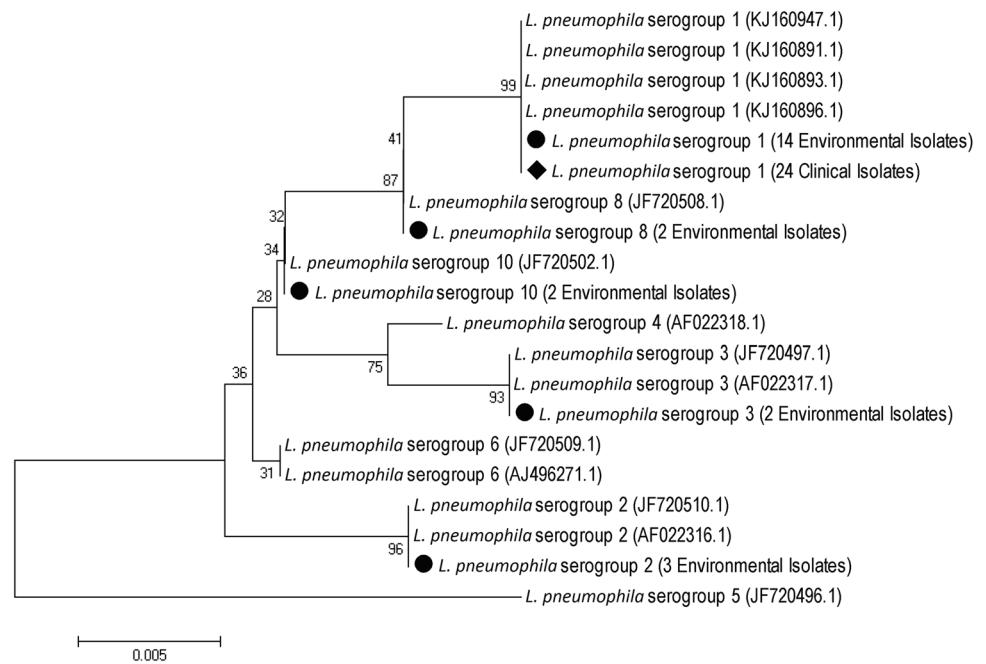
Table 3 Factors associated with *Legionella pneumophila* infectivity

Factors		
Baseline predictors	OR (95% CI)	*P* value
Mean age ± SD	1.164 (1.009–3.089)	0.010*
Gender (male/female)	0.966 (0.939–1.993)	0.742
length of ICU stay	0.951 (0.885–0.998)	<0.001*
Duration of ventilator use	0.921 (0.863–0.984)	<0.001*
Hospital	1.127 (0.756–1.678)	0.445
Smoking	1.895 (1.001–1.995)	0.025*

SD Standard deviation; *statistically significant (<0.05)

based on a systematic collection of both environmental and patient *Legionella* isolates.

To the best of our knowledge, this is the first study from Iran to evaluate the relationship between the serogroup diversity of *L. pneumophila* isolates collected from patients and environmental samples.

L. pneumophila was identified in 8.4% and 19.8% of patients and water resources in our study, respectively. Generally, water supply systems are an important source of infection. According to several reports from Iran, the prevalence of *Legionella* species in water resources is high, and *L. pneumophila* is the most prevalent species (Khaledi et al. 2019; Yaslianifard et al. 2019). The prevalence of this pathogen is estimated at 9.6% in Iranian clinical samples, ranging from 0.4 to 22.1% (Khaledi et al. 2019). According to previous results, this agent could be important for Iranian patients with respiratory problems. Therefore, use of an

efficient diagnostic method can help with a timely treatment of patients' symptoms (Goodarzi et al. 2020).

In the present study, *L. pneumophila* serogroup 1 was responsible for VAP infection. In this regard, several studies have shown that most patients with Legionnaires' disease (LD) were infected with serogroup 1 isolates. These results suggest that serogroup 1 is more virulent than other serogroups (Den Boer et al. 2008). Also, 60.8% of positive samples from water resources belonged to serogroup 1 in our study. Based on other reports from different regions of Iran, serogroup 1 is more frequently found in water resources (Moosavian et al. 2019; Yaslianifard et al. 2019), although it varies in other countries (Den Boer et al. 2008; Harrison et al. 2007). The distribution of *legionella* isolates may depend on the location and geographical area, as shown in a study from Korea (Lee et al. 2010).

Unlike other studies, environmental isolates in our study showed similar *Legionella* genotypes to those collected from the patients. There are two possible explanations for this finding. First, surveys based on standardized questionnaires may not be able to determine the actual source of LD. It is also reasonable to assume that the collection of isolates from the environment represents the country. However, it is feasible that virulent strains should be examined in the air, but not in aquatic sources, as they spread through different sources on days of increased humidity (Fisman et al. 2005). Second, the genotypes of *Legionella,* most commonly isolated from human respiratory secretions, are also present in sampling sources, although their concentration cannot be determined (Den Boer et al. 2008).

In the present study, age, length of ICU stay, duration of ventilator use, and smoking were significantly associated with *L. pneumophila* infectivity. Several studies have shown that the infectivity of *L. pneumophila* is related to the host and different strains. For example, the demographic and clinical characteristics of patients infected with two common isolates (Paris and Lorraine) were different in France. The Paris strain was significantly related to a history of cancer or hematologic malignancy, steroid therapy, and female sex, while it was negatively correlated with smoking. On the other hand, the Lorraine strain was only related to smoking. The crude mortality rate correlated with the Paris strain was higher than that of the sporadic strain, while the Lorraine strain was associated with a lower mortality rate than the sporadic strain (Ananth and Kleinbaum 1997; Ginevra et al. 2009).

The known host risk factors associated with LD include an immunocompromised status, diabetes, older age, being a recipient of a transplant or chemotherapy, smoking, and chronic obstructive pulmonary disease (Farnham et al. 2014). Also, the most important environmental risk factors include proximity to cooling towers, decorative fountains, and whirlpool spas, residence in a healthcare facility, and traveling (Beauté et al. 2013; Greig et al. 2004).

Conclusion

This is the first report from Iran to evaluate differences in the serogroup distribution of environmental and clinical strains of *L. pneumophila*. We found that serogroup 1 was the most common type in the samples. However, further studies in different regions of Iran are warranted to determine the relationship between environmental and clinical strains.

Declarations

Ethics approval and consent to participate This study was carried out at Pasteur Institute of Iran (PII; Tehran, Iran) from May 2017 to March 2018. It was performed according to the Declaration of Helsinki (1975) guidelines and local regulations and also approved by the Ethics Committee of PII. Written informed consent was directly obtained from all of the participants.

Consent for publication Not applicable.

Competing interests The authors declare competing interests.

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