

Legionella pneumophila in Bronchoalveolar Lavage Samples of Patients Suffering from Severe Respiratory Infections: Role of Age, Sex and History of Smoking in the Prevalence of Bacterium

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SUMMARY

Introduction *Legionella pneumophila* is the most commonly detected cause of legionellosis, which is an acute respiratory tract infection with high morbidity and mortality rates.

Objective The aim of this study was to investigate the prevalence rate of *L. pneumophila* in bronchoalveolar lavages and study the role of sex, age and history of smoking as risk factors for susceptibility to the bacterium.

Methods One hundred bronchoalveolar lavage samples were collected from the Iranian health centers and immediately transferred to laboratory. The samples were cultured and those that were *L. pneumophila* positive were subjected to PCR method with respect to the 16S rRNA gene.

Results Twelve out of 100 samples were positive for *L. pneumophila* (12%). Patients older than 70 years had the highest incidence of *L. pneumophila* (17.77%). Prevalence of *L. pneumophila* in male and female patients was 14.81% and 8.69%, respectively. Total incidence of *L. pneumophila* in patients with and without history of smoking was 18% and 6%, respectively. There were significant differences in the incidence of bacterium between groups of our study.

Conclusion Sex, age and history of smoking are predominant risk factors for the occurrence of *L. pneumophila*. However, more studies should be undertaken to confirm these results.

Keywords: *Legionella pneumophila*; prevalence; bronchoalveolar lavages; respiratory infections; risk factors

INTRODUCTION

Respiratory tract infections (RTIs) are the most common, and potentially most severe, of infections treated by health care practitioners. Based on the previous report by Hoyert et al. [1], there were 52,136 deaths attributable to pneumonia in 2011 (16.7% of all deaths). The total number of deaths due to pneumonia in 2010 was 49,597 (16.1% of all deaths) [1]. RTIs are usually caused by viruses, however the roles of bacteria are also significant.

Legionellosis (Legionnaires' disease and Pontiac fever) is an environment related and acute RTI caused by gram-negative, rod-shaped bacteria of the genus *Legionella*. Pontiac fever is a self-limiting influenza-like syndrome; Legionnaires' disease is more severe, has pneumonia as the major clinical finding, and can be fatal. At this time, there are 52 *Legionella* species [2] and 70 serogroups [3]. Of these species, 25 are notorious to cause human infection [2]. Ninety percent of *Legionella* infections in humans are caused by *Legionella pneumophila* (*L. pneumophila*) [4].

L. pneumophila is a gram-negative, fastidious and aerobic bacilli, catalase-positive, heterotrophic, motile, non-fermentative and urease and nitrate negative bacterium [5]. Legionel-

losis cases reported annually increased 217%, from 1,110 in 2000 to 3,522 in 2009, and the crude national incidence rate increased 192%, from 0.39 per 100,000 persons in 2000 to 1.15 in 2009 [6].

The elderly, smokers, persons with chronic respiratory or kidney disease and immune deficient individuals are at greater risk of legionellosis [7]. The disease is the main apprehension of public health professionals and individuals involved with maintaining and constructing water supply systems. The factors that lead to outbreaks or cases of Legionnaires' disease are not completely understood, but certain events are considered prerequisites for infection. These include the presence of the bacterium in an aquatic environment, amplification of the bacterium to an unknown infectious dose, and transmission of the bacteria via aerosol to a human host susceptible to infection [7].

Due to the slow-growing process of the bacterium in specific culture media, which are usually expensive and not readily available, and also due to high risks of coming in contact with the bacterial culture, applications of novel methods are essential in detection and diagnosis of legionellosis. Molecular techniques like the polymerase chain reaction (PCR) targeting the 16S ribosomal RNA of the bacterium have

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been addressed as an accurate, safe, rapid and sensitive methods for the diagnosis of bacterium [8, 9].

OBJECTIVE

As far as we know, there were no previously published data on the distribution of *L. pneumophila* in Iranian health centers. Therefore, the present study was carried out in order to examine the prevalence of *L. pneumophila* in the bronchoalveolar lavage samples of patients suffering from RTIs that were referred to the Iranian health centers.

METHODS

Samples

This cross sectional study was done based on high prevalence of RTIs in patients of some Iranian health centers. From November 2012 to March 2013, a total of 100 bronchoalveolar lavage samples from patients suffering from RTIs were collected using bronchoscopy. The prevalence of RTIs in this period of time in those Iranian health centers was 34%. At the time of sampling, the information about the age, sex and history of smoking of each patient was recorded. Approximately 10 ml of each sample was immediately put into a sterile Falcon™ tube, which was placed inside a flask containing ice and without delay transferred to the Biotechnology Research Center of the Islamic Azad University of Shahrekord.

Bacterial isolation

Prior to culturing, bronchoalveolar lavage 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 αBCYE selective medium agar (Difco Laboratories, Detroit, Mich., USA) and on plates containing L-cysteine (0.44 mg/mL), ferric pyrophosphate (0.250 mg/mL), glycine (3.0 g/L), vancomycin (0.0025 mg/mL) and polymyxin B (0.006 mg/mL), which are named αBCYE-GVP selective agar medium. Plates were incubated at 37°C in a humidified atmosphere without CO₂ during 5 days. Colonies with the typical “ground glass” appearance of Legionella were subcultured on two non-selective media, sheep-blood agar and αBCYE Agar without L-cysteine. Colonies that grew on αBCYE-GVP 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 polyclonal rabbit sera (m-Tech Alpharetta, Ga., USA).

DNA extraction and PCR amplification

L. pneumophila strains were submitted to DNA extraction using a DNA extraction kit (Fermentas, Germany), according to the manufacturer's instructions. The extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell [10]. The extracted DNA of each sample was kept frozen at -20°C until used. Primer sequences used for PCR, *Legionella*-F: 5' GCTAATACCG-CATAATGTCTGAGG-3' and *Legionella*-R: GGTGCT-TCTGTGGGTAACG- 3', were designed from 16S ribosomal RNA gene of *Legionella* (ACCESSION AB811078). PCR amplification was carried out in a total volume of 25 µl in 0.5 ml tubes containing 1 µg of genomic DNA, 1 µM of each primer, 2 mM MgCl₂, 200 µM dNTP, 2.5 µl of 10X PCR buffer and 1 unit of Taq DNA polymerase (Fermentas, Germany). The samples were placed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany) with an initial denaturation step at 95°C for 5 minutes, then amplified for 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, extension at 72°C for 1 minute and final extension step at 72°C for 5 minutes.

Gel electrophoresis

The PCR amplification products (10 µl) were subjected to electrophoresis in a 1% agarose gel in 1X TBE buffer at 80 V for 30 minutes, stained with ethidium bromide, and images were obtained in a UVIdoc gel documentation systems (UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany).

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* between various studied groups of patients. A p-value <0.05 was considered statistically significant.

Ethical issues

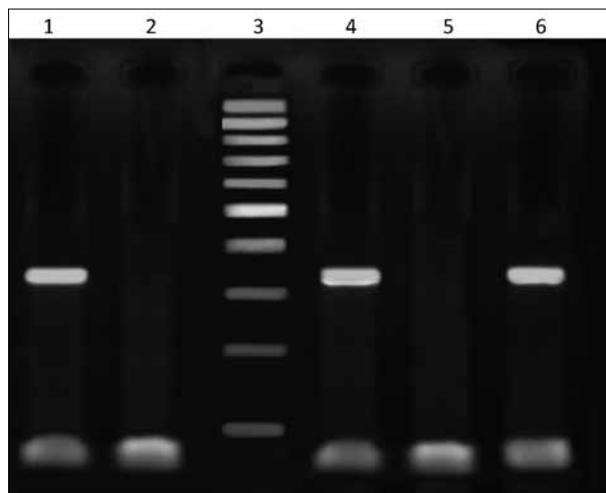
The present study was authorized by the ethical committee of the education health care centers of the Shahrekord city, Iran, and the Biotechnology Research Center of the Islamic Azad University of Shahrekord Branch, Iran. All patients or their parents signed the written informed consent.

RESULTS

A total of 100 bronchoalveolar lavage samples were subjected to the culture technique. Distribution of *L. pneumophila* in the studied groups is shown in Table 1. Of 100

Table 1. Distribution of *L. pneumophila* in the bronchoalveolar samples of patients with respiratory infections

Age (year)	No. of samples collected	Distribution of <i>L. pneumophila</i> (%)
≤30	4	-
31–50	18	1 (5.55)
51–70	33	3 (8.57)
≥71	45	8 (17.77)
Total	100	12 (12)

**Figure 1.** Results of gel electrophoresis for identification of *L. pneumophila* in bronchoalveolar samples

Lanes: 1 – Positive control; 2 – Negative control; 3 – 100 bp DNA ladder (Fermentas, Germany); 4 – Positive samples for *L. pneumophila* (337 bp band); 5 – negative samples for *L. pneumophila*; 6 – Positive samples for *L. pneumophila* (337 bp band)

Table 2. Distribution of *L. pneumophila* in the bronchoalveolar samples of hospitalized male and female patients with and without history of smoking

Types of broncho alveolar samples		No. of samples collected	Distribution of <i>L. pneumophila</i> (%)
Male	With smoking	30	6 (20)
	Without smoking	24	2 (8.33)
	Total	54	8 (14.81)
Female	With smoking	20	3 (15)
	Without smoking	26	1 (3.84)
	Total	46	4 (8.69)
Total	With smoking	50	9 (18)
	Without smoking	50	3 (6)
	Total	100	12 (12)

bronchoalveolar lavage samples, 12 samples (12%) were positive for *L. pneumophila* (Table 1). The results of the culture technique were confirmed by the PCR method using the specific primers for the 16S rRNA gene of *L. pneumophila* (Figure 1).

We found that patients older than 70 years had the highest incidence of *L. pneumophila* (17.77%), while 30–50-year-old patients had the lowest incidence (5.55%). On the other hand, there were no positive results for patients below the age of 30. There were significant differences ($p=0.043$) in the incidence of *L. pneumophila* between old and young patients.

Distribution of *L. pneumophila* in the bronchoalveolar lavage samples of male and female patients with and with-

out history of smoking is shown in Table 2. Distribution of *L. pneumophila* in male and female patients was 14.81% and 8.69% respectively, but there were no statistically significant differences in the incidence of *L. pneumophila* between male and female patients. Total distribution of *L. pneumophila* in patients with and without history of smoking was 18% and 6%, respectively. We found a statistically significant ($p=0.041$) association between the incidence of *L. pneumophila* in patients with history of smoking and those without it.

DISCUSSION

As far as we know, the present study was the first clinical report on the distribution of *L. pneumophila* in RTIs with respect to sex, age and history of smoking in Iran. The results of our investigation showed high prevalence of *L. pneumophila* in bronchoalveolar lavage samples of patients with RTIs (12%). Total prevalence of *L. pneumophila* in the hospital samples of Ghotaslou et al. [11], Chaudhry et al. [12], Yu et al. [13] and Azara et al. [14] was 2.85%, 13%, 63% and 46.66%, respectively. These investigations highlight large differences in the prevalence of *L. pneumophila*. This could be related to differences in the type of sample (bronchoalveolar lavage, hospital water, stool, blood, urine, and other clinical samples) tested, number of samples, method of sampling, history of patients (with and without smoking history), season of sampling, experimental methodology, geographical area, and climate differences in the areas where the samples were collected, which would have differed between each study.

Our results showed that main causes of the RTIs in 88 patients (88%) of our investigation were not related to *L. pneumophila*. Based on the Iranian literature, *Klebsiella pneumoniae* (48.6%) [15], *Acinetobacter* spp. (33.7%) [15], respiratory syncytial virus (22.2%) [16], influenza virus (10.9%) [17], *Mycoplasma pneumoniae* (10%) [17], parainfluenza virus (15.8%) [17] and adenovirus (5.9%) [17] were the most commonly isolated pathogens. Therefore, they may be the causative agents of RTIs in those 88 patients. Total prevalence of *L. pneumophila* of our survey was entirely lower than those from *Klebsiella pneumoniae* (48.6%) and *Acinetobacter* spp. (33.7%) [15], as well as from the respiratory syncytial virus (22.2%) [16], but was higher than the viral agents described by Farshad et al. [17].

To our best knowledge, our results concerning the presence of *L. pneumophila* in the bronchoalveolar lavage samples are the highest prevalence reports of this bacterium in the clinical samples in the world. One possible explanation for the high prevalence of *L. pneumophila* in study region is that climatic variables such as heat, thunderstorms and rain, together with variable barometric pressure, may have affected the patients' autonomic nervous system. These variables could affect immunity, thus making people more susceptible to infections. Alternatively, the higher prevalence of *L. pneumophila* may be related to cold climate of the study region. The samples of our study were also collected from November to March, which are the cold

months of the year in Iran. Herrera-Lara et al. [18] suggested that the highest incidence of community acquired pneumonia was in the cold seasons of the year.

The seasonal aspect of the illness has to do with the fact that its incidence increases systematically during a particular season of the year. In order for hospital services to be planned more effectively and for the pathogenesis of the disease to be better understood, it is essential to know this relationship. Therefore, the highest levels of health care should be attained during the colder months of the year.

Another part of our investigation highlights the age- and sex-dependent distribution of *L. pneumophila*. The results of our investigation showed that age and sex of patients are predisposing factors for prevalence of *L. pneumophila*. Men usually have more contact with the external contaminated environment. They work outdoors, while women usually stay at home and are not in close contact with contaminated environments. Therefore, it is clear that the prevalence of *L. pneumophila* in men (14.81%) was higher than that in women (8.69%).

Our results also showed that patients older than 70 years had the highest incidence of *L. pneumophila* (17.77%), while the 30–50-year-old patients had the lowest incidence (5.55%). Nagalingam et al. [19] reported that of a total of 123 serum samples of patients with RTIs, 39 (31.7%) were positive for *L. pneumophila* IgM/G/A, while 2 samples (1.6%) were positive for IgM only. Individual hospital environments, sex and ethnicity did not significantly ($p>0.05$; chi-squared) affect the seroprevalence of *L. pneumophila*. Sopena et al. [20] showed that elderly patients with community-acquired pneumonia caused by *L. pneumophila* had a higher frequency of underlying comorbidities and presented less frequently with fever and classical non-respiratory symptoms and laboratory abnormalities of Legionnaires' disease than younger patients, although greater severity of illness at onset and higher mortality were not significantly different between the two age groups. Our results represented that the total prevalence

of *L. pneumophila* in patients with history of smoking was 18%, which was entirely higher than in those who had no smoking history (6%). The respiratory status of patients with history of smoking could have been markedly impaired even before *Legionella* infection, leading to an earlier and more severe respiratory failure. In total, Ngeh and Goodbourn [21] indicated that age, sex, hypertension, smoking, diabetes and ischemic heart disease are risk factors in the occurrence and prevalence of *L. pneumophila*. In an overall view, age, sex and history of smoking are predisposing and/or risk factors for the prevalence of *L. pneumophila* in patients suffering from RTIs.

As it was shown, legionellosis is considered to be one of the most important causes of community-acquired RTIs around the world. Therefore, rapid, safe and sensitive diagnosis of its causative agents is essential in the control and eradication of diseases. Culture of *L. pneumophila* from clinical specimens is not an appropriate method for its isolation and identification due to the long period of incubation and its dangerous procedure [22]. Our study showed that application of specific primers which were designed based on the 16S rRNA gene of the *L. pneumophila* is sufficient for rapid and safe diagnosis of bacterium in clinical samples.

CONCLUSION

In conclusion, we identified a large number of positive samples (12%) in *L. pneumophila* isolated from bronchoalveolar lavage samples of patients with RTIs in Iranian health centers. Marked sex- and age-specific variations in the distribution of *L. pneumophila* were also found. The impact of history of smoking should not be denied in the prevalence of *L. pneumophila*. However, more studies in larger groups of *L. pneumophila* strains are necessary to confirm these findings. We suggest the application of PCR based 16S rRNA gene of the *L. pneumophila* for its rapid, safe and accurate diagnosis.

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***Legionella pneumophila* у бронхоалвеоларним узорцима лавата особа оболелих од тешких респираторних инфекција: улога узраста, пола и навике пушења дувана на учесталост јављања бактерије**

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КРАТАК САДРЖАЈ

Увод *Legionella pneumophila* је најчешћи узрочник легионелозе, акутне инфекције дисајног тракта, чија је стопа морбидитета и морталитета висока.

Циљ рада Циљ овог истраживања је био да се утврди стопа преваленције бактерије *L. pneumophila* у бронхоалвеоларним лаватима и испита улога узраста, пола и навике пушења дувана (у анамнези) као фактора ризика за подложност овој бактерији.

Методе рада Прикупљено је 100 узорака бронхоалвеоларних лавата из здравствених центара Ирана који су одмах упућени у лабораторију. Узорци су засејани на хранљиве подлоге, а они за које би се утврдило да су позитивни на *L. pneumophila* подвргавани су методи *PCR* у вези с геном *16S rRNA*.

Резултати Дванаест од 100 узорака (12%) било је позитивно на бактерију *L. pneumophila*. Инциденција бактерије је била највиша код болесника старијих од 70 година (17,77%). Преваленција код болесника мушког и женског пола била је 14,81%, односно 8,69%. Укупна инциденција бактерије код болесника који су пушили била је 18%, док је код оних који нису била 6%. Постојале су знатне разлике у инциденцији бактерије међу испитиваним групама.

Закључак Пол, узраст и навика пушења дувана су главни фактори ризика за јављање бактерије *L. pneumophila*. Међутим, потребне су додатне студије, како би се ови резултати потврдили.

Кључне речи: *Legionella pneumophila*; преваленција; бронхоалвеоларни лавати; респираторне инфекције; фактори ризика

Примљен • Received: 04/07/2014

Прихваћен • Accepted: 20/10/2014