

Antimicrobial Resistance Properties of *Legionella pneumophila* Isolated From The Cases of Lower Respiratory Tract Infections

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ABSTRACT

Legionella pneumophila is one of the main pathogenic agents responsible for pneumonia and respiratory tract infections. It has high levels of resistance against commonly used antibiotics. The present investigation was carried out to study the prevalence and antibiotic resistance pattern of *L. pneumophila* strains isolated from patients suffered from RTIs. Totally, 250 respiratory samples were selected and immediately tested. All samples were cultured and those that were positive for *L. pneumophila* were subjected to PCR and disk diffusion. Twenty-seven out of 250 respiratory samples (10.80%) were positive for *L. pneumophila*. Results were also confirmed by *lepA* gene-based PCR amplification. Prevalence of *L. pneumophila* in male and female patients were 13.84% and 7.50%, respectively ($P < 0.01$). Older than 60 years old patients had the highest prevalence of infection with *L. pneumophila* ($P < 0.05$). Bacterial strains harbored the highest levels of resistance against ciprofloxacin (81.48%), erythromycin (77.77%), clarithromycin (51.85%) and moxifloxacin (48.14%), while prevalence of resistance against rifampicin (18.51%), doxycycline (22.22%) and azithromycin (25.92%) was low. Primary identification of *L. pneumophila* positive strains and their regular treatment with rifampicin, doxycycline and azithromycin can reduce the risk of transmission and spread of *L. pneumophila*.

Keywords: *Legionella pneumophila*, Prevalence, Antibiotic resistance pattern, Respiratory tract infection.

INTRODUCTION

Respiratory tract infections (RTIs) are one of the most common and severe types of infectious diseases all-around the world. Documented data revealed that more than 16% of death are occurred due to the RTIs (1,2). RTIs and pneumonia are responsible for more than 50,000 cases in 2010 (1,2). RTIs accounted for about 44,000 hospital admissions with an average length of stay of 6.3 days^{1,2}. RTIs are usually caused by viruses, however the roles of bacteria are also significant.

Among all bacterial agents which were isolated from the cases of RTIs and pneumonia, *Legionella* species (*Legionella* spp.) are one of the most commonly considered pathogens³⁻⁵. Among all species of *Legionella*, *Legionella pneumophila* (*L. pneumophila*) has the highest clinical importance⁶⁻⁸. It is a causative agents of human legionellosis or Legionnaires Disease (LD) and community-acquired and nosocomial pneumonia⁸⁻¹⁰. LD is responsible for more than 18,000 cases of hospitalization in developed countries^{10,11}. RTIs and pneumonia caused by *L. pneumophila* are usually

known by confusion, fever, headache, diarrhea, abdominal pain, chills, non-productive cough and myalgia⁶⁻¹¹.

RTIs and pneumonia caused by this bacterium often required antibiotic therapy; However, antibiotic resistant strains of this bacterium cause more severe and dangerous diseases for longer periods of time than susceptible strains^{12,13}. According to the recent epidemiological studies, *L. pneumophila* strains show a high prevalence of resistance (50-100%) against commonly used antibiotics including tigecycline, ceftriaxone, rifampicin, azithromycin, erythromycin, moxifloxacin, ciprofloxacin, levofloxacin, doxycycline and clarythromycin^{12,13}.

According to the uncertain role of *L. pneumophila* strains as a causative agent of RTIs in male and female of various ages caused us to do this investigation with respect to study the distribution of *L. pneumophila* in the respiratory samples taken from patients suffered from RTIs as well as study the antimicrobial resistance pattern of bacterial isolates against 10 commonly used antibiotics used for RTIs.

MATERIALS AND METHODS

Samples collection and bacterial isolation

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 250 respiratory samples were randomly selected and analyzed for presence of *L. pneumophila*. At the time of sampling, information about the age, sex and clinical symptoms of the patients were recorded. Ten ml of each sample was immediately transferred to a sterile falcon tube containing ice and was immediately transferred to the laboratory.

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⁻¹), ferric pyrophosphate (0.250 mg mL⁻¹), glycine (3.0 gL⁻¹), vancomycin (0.0025 mg mL⁻¹) and polymyxin B (0.006 mgmL⁻¹), which are named aBCYE-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 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) (14). The extracted DNA of each sample was kept frozen at -20°C until used. Primer sequences used for PCR, Legionella-F: 5'-GTTGGGCACTACAGTTATCTCTTC-3' and Legionella-R: GTTAGTTACTACGGT TTCAATACG AC-3' (354 bp) were designed from *lepA* gene of Legionella. PCR reactions were performed in a total volume of 25 µL, including 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 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 50 s, annealing at 59°C for 1 min, extension at 72°C for 1 min and final extension step at 72°C for 5 min. 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.

Antibiotic susceptibility test

L. pneumophila strains of lower respiratory tract infections were cultured on aBCYE selective medium agar (Difco Laboratories, Detroit, Mich., USA). Antimicrobial resistance of the *L. pneumophila* strains against 10 commonly used antibiotics was determined using the instruction of Clinical and Laboratory Standards Institute guidelines (15). Susceptibility of *L. pneumophila* isolates were tested against ceftriaxone (30 µg/disk), azithromycin (15 µg/disk), erythromycin (15 µg/disk), ciprofloxacin (5 µg/disk), doxycycline (30 µg/disk), rifampicin (5 µg/disk), tigecycline (15 µg/disk), moxifloxacin (5 µg/disk), clarythromycin (2 µg/disk) and levofloxacin (1 µg/disk) antimicrobial agents (Oxoid, UK). Plates containing the discs were allowed to stand for at least 30 min before incubated at 37°C in a humidified atmosphere without CO2 during 5 days. The diameter of the zone of inhibition produced by each antimicrobial disc was measured and interpreted using the CLSI zone diameter interpretative standards (15). *L. pneumophila* ATCC 33152 and *S. aureus* ATCC 25923 were used as quality control organism in antimicrobial susceptibility determination.

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 antibiotic resistance between various studied groups of patients. A P value < 0.05 was considered statistically significant.

RESULTS

Table 1 represents the total prevalence of *L. pneumophila* in the samples taken from patients suffered from RTIs. We found that 27 out of 250 samples (10.80%) were positive for *L. pneumophila*. Results of the culture method were also confirmed using the *lepA* gene-based PCR amplification (figure 1). Total prevalence of *L. pneumophila* in the male and female patients suffered from RTIs were 13.84% and 7.50%, respectively. Statistically significant differences were seen for the prevalence of *L. pneumophila* between male and female (P < 0.01) and old and young patients (P < 0.05).

Antibiotic resistance properties of *L. pneumophila* strains isolated from samples taken from patients suffered from RTIs is shown in table 2. *L. pneumophila* strains of our investigation

Table 1: 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 (%)
Male	<20 years	30	2 (6.66)	2 (6.66)
	20-40 years	32	4 (12.50)	4 (12.50)
	40-60 years	33	5 (15.15)	5 (15.15)
	>60 years	35	7 (20)	7 (20)
	Total	130	18 (13.84)	18 (13.84)
Female	<20 years	26	1 (3.84)	1 (3.84)
	20-40 years	31	2 (6.45)	2 (6.45)
	40-60 years	29	2 (6.89)	2 (6.89)
	>60 years	34	4 (11.76)	4 (11.76)
	Total	120	9 (7.50)	9 (7.50)
Total	250	27 (10.80)	27 (10.80)	

harbored the highest levels of resistance against ciprofloxacin (81.48%), erythromycin (77.77%), clarithromycin (51.85%) and moxifloxacin (48.14%). Bacterial strains which were isolated from male patients harbored the higher prevalence of antibiotic resistance than female ($P < 0.05$). *L. pneumophila* strains harbored the lowest levels of resistance against rifampicin (18.51%), doxycycline (22.22%) and azithromycin (25.92%).

DISCUSSION

The results of the present investigation revealed that resistant strains of *L. pneumophila* had the high ability for presence as a causative agent of the RTIs in Iranian patients. Totally, 10.80% of samples were positive for *L. pneumophila* which

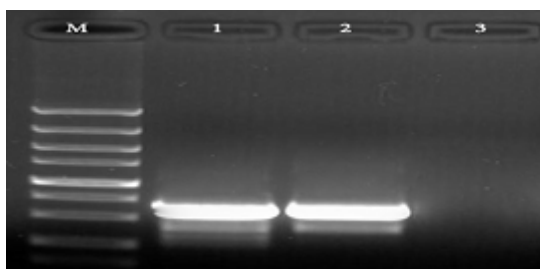


Fig. 1: Results of the gel electrophoresis of PCR products used for amplification of *lepA* gene of *L. pneumophila* isolated from patients suffered from RTIs. M: 100 bp ladder, 1: Positive sample (354 bp), 2: Positive control and 3: Negative control

was considerable high. We found that the prevalence of bacteria in male and female patients were 13.84% and 7.50%, respectively. A possible clarification for the higher prevalence of *L. pneumophila* in male than female is that men usually have more contact with the contaminated external environment. They work outside the house but women usually stay at home and are not in close contact with outside. In fact, most of the Iranian women prefer to work at home. In addition, higher levels of immunity in men than women caused to their infection with resistant strains of *L. pneumophila*. We also found that older patients had the higher prevalence of bacteria than younger which may be due to their low levels of immunity.

Several investigations were conducted in this field al-around the world. Khedri et al. (2015) (14) reported that of 150 samples tested for presence of *L. pneumophila*, 18 samples (12%) were positive. They showed that the prevalence of bacteria in male and female patients were 14.40% and 8.30%, respectively. They also showed that older patients had the higher prevalence of *L. pneumophila* which was completely similar to our findings. Total prevalence of *L. pneumophila* in the clinical samples of Chaudhry et al. (2000) (16), Yu et al. (2008) (17), Ghotaslou et al. (2013) (18) and Azara et al. (2006) (19) were 5.1%, 13%, 2.85% and 26.66%, respectively. Ngeow et al. (2005) (20) were analyzed 1800 patients for presence of respiratory pathogens. They showed that *L. pneumophila* is one of the most commonly detected

Table 2: Antibiotic resistance pattern of *Legionella pneumophila* isolated from the respiratory samples taken from patients suffered from RTIs

Samples (No. positive)	Antibiotic resistance pattern (%)									
	Cef*	Azi	Ert	Cip	Dox	Rif	Tig	Mox	Clar	Lev
Male (18)	8 (44.44)	5 (27.77)	15 (83.33)	16 (88.88)	4 (22.22)	4 (22.22)	8 (44.44)	9 (50)	10 (55.55)	8 (44.44)
Female (9)	3 (33.33)	2 (22.22)	6 (66.66)	6 (66.66)	2 (22.22)	1 (11.11)	3 (33.33)	4 (44.44)	4 (44.44)	3 (33.33)
Total (27)	11 (40.74)	7 (25.92)	21 (77.77)	22 (81.48)	6 (22.22)	5 (18.51)	11 (40.74)	13 (48.14)	14 (51.85)	11 (40.74)

*Cef: ceftriaxone (30 µg/disk), Azi: azithromycin (15 µg/disk), Ert: erythromycin (15 µg/disk), Cip: ciprofloxacin (5 µg/disk), Dox: doxycycline (30 µg/disk), Rif: rifampicin (5 µg/disk), Tig: tigecycline (15 µg/disk), Mox: moxifloxacin (5 µg/disk), Clar: clarythromycin (2 µg/disk), Lev: levofloxacin (1 µg/disk).

pathogens in studied samples. Similar findings have been reported previously by Nagalingam et al. (2005) (21) and Amemura-Maekawa et al. (2010) (22).

These large differences which were found for the prevalence of *L. pneumophila* in various researches maybe due to the differences in the type of sample (bronchoalveolar lavage, urine, blood, water, stool, and other clinical samples) tested, number of samples, method of sampling, history of patients (with and without smoking history or other predisposing factors), 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.

We found that bacterial strains harbored the highest levels of resistance against ciprofloxacin, erythromycin, clarithromycin and moxifloxacin. These are mainly used for treatment of infections caused by Gram-negative bacteria. Therefore, it showed that treatment of RTIs in Iranian health centers were done according to the results of the disk diffusion and mainly based on the results of Gram staining. The main causes for the high prevalence of resistance against these antibiotics are their irregular, excessive and unauthorized prescription. Several investigations were conducted on the prevalence of antibiotic resistance in *L. pneumophila* strains of environmental and clinical samples. Moffie and Mouton (1988) (23) reported the low levels of *L. pneumophila* resistance against rifampicin, erythromycin, norfloxacin and ciprofloxacin. In fact, these antibiotic agents were

effective for treatment of RTIs caused by *L. pneumophila* on 1988 year. Excessive and irregular prescription of these antibiotics caused increase in the levels of resistance such that showed in our results. De Giglio et al. (2015) (13) reported that the levels of minimum inhibitory concentration of azithromycin, ciprofloxacin, levofloxacin, moxifloxacin, and tigecycline were significantly lower than other tested antibiotics. They also showed that doxycycline, tigecycline and cefotaxime are effective antibiotic agents for clinical strains of *L. pneumophila*. Malleghol et al. (2014) (24) reported similar results for the antibiotic resistance of *L. pneumophila* strains of clinical samples. High differences which were found in the prevalence of resistance against antibiotics are mainly due to the availability of antibiotics, idea of medical practitioners to prescription of antibiotics, cost of antibiotic agents and also status and conditions exist for prescription of antibiotics.

CONCLUSIONS

In conclusion, we identified a large numbers of *L. pneumophila* in the respiratory samples of male and female patients of various age groups suffered from RTIs as well as their antibiotic resistance pattern. We found that the highest levels of health monitoring should be done for older than 60 years old male patients. We found that judicious and regular prescription of rifampicin, doxycycline and azithromycin can control the risk of RTIs due to the *L. pneumophila*. We recommended using from simple disk diffusion method to determine proper antibiotic agents for treatment of cases of RTIs due to this bacterium.

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