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Detection of the Legionnaires' Disease Agent in Patients With Respiratory Symptoms by Culture, Detection of Urinary Antigen and Polymerase Chain Reaction of the *16S rRNA* Gene in Ahvaz, Iran

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Abstract

Background: Legionnaires' disease (LD) is a common form of severe pneumonia, caused by *Legionella* spp. *Legionella pneumophila* is an important agent of severe pneumonia including 15 serogroups, which are all human pathogens. However, *L. pneumophila* serogroup 1 is the most prevalent agent of LD. Fatality rates among elderly and immunocompromised patients are high and may occur as a result of infection with this pathogen.

Objectives: The aim of this study was to detect the LD agent in clinical samples of patients with respiratory symptoms by culture, urinary antigen and polymerase chain reaction (PCR) of the *16SrRNA* gene.

Methods: In this study, a total of 200 specimens (including 100 urine and 100 respiratory samples), which were collected from hospitalized patients with respiratory symptoms were examined. The respiratory specimens were inoculated to the buffered charcoal-yeast extract and modified Wodowsky and Yee agar media for isolation of the *Legionella* spp. The *16S rRNA* gene in the respiratory specimens was amplified by the PCR method and the urinary antigen of *L. pneumophila* serogroup 1 was detected by EIA (enzyme immunoassay) test using the Coris *Legionella* V-test kit.

Results: From a total of 200 specimens from patients with respiratory symptoms, 5% of urine specimens and 3% of respiratory specimens were positive for *L. pneumophila* using the EIA test and PCR of the *16SrRNA* gene, respectively. The results of the culture of the respiratory samples showed that 1% of them were positive for *Legionella* spp.

Conclusions: In this study, the LD agent was detected by the rapid EIA test. In addition, the sensitivity of the urinary antigen test using the Coris *Legionella* V-test kit for detection of *L. pneumophila* in respiratory specimens was more than those of the PCR and culture methods.

Keywords: Legionnaires' Disease, Polymerase Chain Reaction, *16S rRNA*, *Legionella pneumophila*

1. Introduction

Legionella species are facultative intracellular Gram-negative bacilli that present in natural water sources, man-made water systems, and pot humid soil. These bacteria could infect and multiply in both phagocytic protozoa and within mammalian professional phagocytes and epithelial cells (1). Up to now, 53 species and 70 serogroups of Legionellaceae have been described (2), which *Legionella pneumophila* serogroup O1 has been known as the most prevalent of Legionnaires' disease (LD) among the 15 recognized *L. pneumophila* serogroups (3). The reports have shown that 1% - 5% of community acquired pneumonia (CAP) as well as up to 30% of nosocomial acquired pneumonia caused by *Legionella* species (4, 5).

Legionnaires' disease may occur in the community or in hospitals. The mortality rate can approach 50% in immunocompromised patients (6). Pontiac fever is a mild, self-limiting flu-like illness caused by *Legionella* species but unlike LD, Pontiac fever does not involve the lower respiratory tract (7). There are many risk factors, which have been associated with LD and some of them are age, chronic lung disease, diabetes, malignancy, immunosuppression, immunocompromised diseases and smoking (8). Since the pneumonia caused by *Legionella* does not show the unique presentation, so for confirmation of the LD the laboratory tests are necessary. Although *Legionellae* culture remains the gold standard among the diagnostic methods for LD, the sensitivity of the culture method for *Legionella* species has been estimated to be approximately 60%, which de-

pends on the type of clinical samples (9). *Legionella* are fastidious, slow growing bacteria (colonies appear after 3 to 4 days) and require to be cultured in selective and nonselective media (10).

Polymerase chain reaction (PCR) is a high sensitive and faster method than culture, which has been used for detection of the *Legionellae* bacteria in specimens from the lower respiratory tract (11). However, the rapid diagnosis of LD by detection of the soluble *L. pneumophila* serogroup 1 antigen in urine samples is effective in early treatment decisions. The urinary antigen (UAG) test has proven to be the much sensitive and easy diagnostic method. *Legionella* antigen in urine is detectable one day after onset of the disease, and persists for days to weeks (9). Peptidoglycan-associated lipoprotein (PAL) of *L. pneumophila* is a conserved antigen among *Legionella* species and is considered as a powerful diagnostic antigen in urine (12).

2. Objectives

The aim of this study was to detect the LD agents in clinical specimens of patients with respiratory symptoms referred to Ahvaz teaching hospitals by culture, urinary antigen and PCR of the *16SrRNA* gene.

3. Methods

In this study, a total of 200 specimens (100 urine and 100 respiratory samples, including 94 sputum samples and 6 pleural fluid specimens) were collected from patients hospitalized in Ahvaz teaching hospitals (65 specimens from Imam Khomeini Hospital and 25 specimens from Razi hospital) between May 2015 and January 2016.

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (code: IRAJUMS. RES. 1394. 263). Informed consent was obtained from all patients before the study began. Clinical examinations of the patients by an infectious disease specialist showed that the patients were affected to acute respiratory symptoms (20 cases), pneumonia (71 cases), and bronchiectasis (9 cases).

3.1. Culture

Each respiratory specimen was aliquoted in two parts, one part inoculation to the culture media and another part was stored at -70°C until PCR accomplishment. The sputum specimens were decontaminated by heat treatment (56°C for 30 minutes), pleural fluid samples were concentrated by centrifugation (13, 14).

The prepared specimens were inoculated to a nonselective media as the buffered charcoal yeast extract (BCYE) agar and a selective media as modified Wodowsky and Yee (MWY) with the supplementary materials (Mast Group Ltd., Merseyside., UK). The plates were incubated at 37°C and about 90% humidity in 3% - 5% CO_2, for up to 2 weeks. All cultures were inspected daily. The *Legionella* suspected colonies were examined by Gram staining, catalase and oxidase tests and then the Gram-negative bacilli were inoculated to the MWY agar, blood agar (Merck, KGaA, Darmstadt, Germany), and eosin methylene blue (EMB) Agar (Merck, KGaA, Darmstadt, Germany). Colonies that grew on the MWY agar but not on the blood agar and EMB agar were identified as *Legionella* spp. (13).

3.2. Extraction of DNA from Respiratory Samples

DNA extraction was performed with the high pure PCR template preparation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The concentration of the extracted DNA was measured by a photobiometer (Eppendorf, Germany) in 260/280 nm UV long waves.

3.3. DNA Amplification

The PCR master mix was prepared in each 50 μL reaction containing 5 μL in 10X PCR buffer, 1 μL of dNTP mix (10 mM), 3 μL $MgCl_2$ (50 mM), 2μL of each primer (10 μM) TAG, A/S Denmark (Table 1), 0.5 μL of Taq DNA polymerase (5 U/μL), 5 μL of DNA template and 31.5 μL of distilled water.

Table 1. Primers of the *16S rRNA* Gene of *Legionella pneumophila*

Gene	Primer Sequences	Product Size, bp	References
16S rRNA	JFP-5´ -AGGGTTGATAGGTTAAGAGC-3´	386-bp	(13)
	JRP-5´- CCAACAGCTAGTTGACATCG-3´		

DNA amplification was performed in a thermocycler (Eppendorf, Germany) under conditions of initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and a final step of extension at 72°C for 5 minutes.

3.4. Electrophoresis

The PCR product was electrophoresed on 1.5% agarose gel (SinaClon BioScience Co, Iran) in 1X buffer Tris/ borate/ EDTA buffer (SinaClon BioScience Co, Iran) at 120V for 60 minutes. The DNA was stained with the DNA safe stain (SinaClon BioScience Co, Iran) and photography of DNA amplified was performed in a gel documentation system (Viber company, French). In this study, *L. pneumophila*

serogroup 1 (ATCC 33152) and distilled water were used as positive and negative controls, respectively.

3.5. Detection of Urinary Antigen

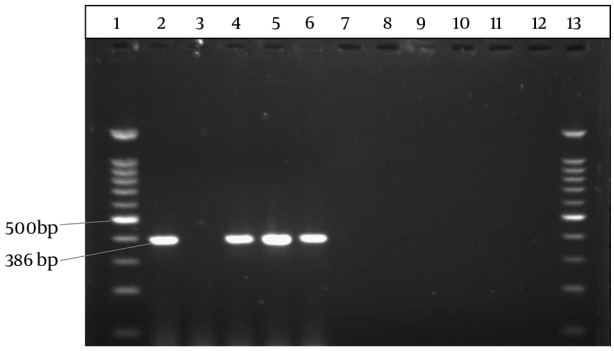
The urine specimens were analyzed for the detection of urinary antigens of *L. pneumophila* serogroup 1 using a commercially kit (*Legionella* V-Test, Coris BioConcept, Gembloux Belgium) according to the manufacturer's instructions. When the urine specimen migrates across the collector pad, the conjugate is rehydrated and migrates alone with the sample. Positive specimens react with the anti- *L. pneumophila* conjugate to form a red line at the test line region of the strip (Upper line). The results were read within 15 minutes.

4. Results

In this study, a total of 200 specimens (including 100 urine and 100 respiratory samples) collected from patients with acute respiratory symptoms (59 males and 41 females) were examined. The mean age of the patients was 54 years (range 15 to 93 years).

The results of this study showed that 5% of the urine specimens and 3% of the respiratory specimens were positive for *L. pneumophila* using the Coris *Legionella* V-test kit and PCR of *16S rRNA* gene, respectively. However, *Legionella* spp. strains were isolated from 1% of the respiratory samples by culture. Based on these results, LD was detected in 5 patients (3 males and 2 females) by the urinary antigen test, while the culture and PCR results of respiratory samples were positive for 1% and 3% of these patients, respectively (Figure 1).

Figure 1. The electrophoresis of the PCR product of the *16S rRNA* gene related to *L. pneumophila* in the sputum samples



Lanes 1 and 13, 100 bp DNA size marker (SinaClon BioScience Co, Iran); lane 2, positive control: *L. pneumophila* ATCC 33152; Lane 3, negative control: distilled water; lanes 4 - 6, Positive samples, and lanes 7 - 12, negative samples.

The results of this study showed that of 75 patients hospitalized in Imam Khomeini hospital, Ahvaz, 4 patients had LD and the presence of *L. pneumophila* in the urine of these patients was validated by the urinary antigen test. However, the identification of *Legionella* were confirmed by PCR in only 2 of these patients, while their culture results were negative. Also, from a total of 25 patients hospitalized in Razi Hospital, Ahvaz, 1 patient had LD and the presence of *Legionella* in the sputum and urine sample of the patient was confirmed by the culture, PCR and urinary antigen test (Table 2).

5. Discussion

Legionella species are ubiquitous in water sources and transmitted via water through the inhalation of contaminated aerosol of water (15). Legionnaires' disease is a severe pneumonia but these infections are seldom recognized. Also, LD occurs both sporadically and in epidemic forms, and if treated improperly can lead to increased morbidity and mortality (12).

Isolation of *Legionella* species from clinical specimens by culture has been known as the gold standard test for many years (13). *Legionella* species are nutritionally fastidious and not easily growing on the culture media. They require the growth factors such as cysteine and Fe^{++} ion, which should be added to the media (7). Culture diagnosis requires proper processing of samples, selective medium, and technical skill (5).

Polymerase chain reaction is a very useful method for the detection of *Legionella* spp. (16). However, differentiation between living and dead *Legionella* cells is not possible by the PCR techniques (17). The detection of soluble *Legionella* antigen in patient's urine was discovered after outbreak of the severe acute pneumonia in Philadelphia convention in 1976 (18), which has been found significant in recent years (9). Urine antigen testing is very useful for patients who cannot produce sputums. The sensitivity and speciality of introduced techniques for detection of the urinary antigen of *L. pneumophila* serogroup1 have reported variable between 70% - 100% (for sensitivity), and 100% (for speciality) (12).

Chen et al. (2015) showed that the sensitivity of the used tests for detection of *L. pneumophila* in clinical specimens was highest for UAG, PCR and culture tests, respectively (19). Our results showed that rapid detection of *L. pneumophila*, serogroup1 as the agent of LD is possible by the Coris *Legionella* V-test kit with the highest sensitivity. However, detection of the *16SrRNA* gene of *L. pneumophila* by PCR demonstrated the good sensitivity (60%), which was more than the culture and isolation of *Legionella* (25%) from clinical specimens. Lower sensitivity of the culture in

Table 2. Frequency of Patients with the Legionnaire's Disease Based on Isolation and/or Identification of *Legionella pneumophila* by Culture, PCR and UAG Methods

Hospital	Patients	Culture	PCR	UAG
Imam Khomeini	75	0	2 (2.7%)	4 (5.3%)
Razi	25	1 (4%)	1 (4%)	1 (4%)
Total	100	1 (1%)	3 (3%)	5 (5%)

Abbreviations: PCR, polymerase chain reaction; UAG, urinary antigen.

detection of the LD agent in clinical specimens might be related to the antibiotic therapy of the patients (20).

Bencini et al. (2007) reported Legionellosis can be diagnosed by PCR of the sputum specimens. This report showed that the *16SrRNA* gene PCR is preferred for the detection of LD caused by *L. pnemophila* (21). Comparison between PCR and culture in diagnosis of the *L. pneumophila* in bronchoalveolar lavage fluid specimens was reported by Hajia et al. (2004). This study showed one positive for the culture and four for the specific *Legionella* PCR (20).

The specificity of three different methods for detection of the *L. pneumophila* in our study was 100%. Statistical analysis (SPSS version 21, and the Chi-square test) showed a significant difference ($P < 0.05$) between UAG, PCR and culture results of total clinical specimens, which obtained from Imam Khomeini and Razi hospitals. There was no significant difference ($P > 0.05$) between LD and variables such as age and gender in our study. Of the 5 patients with positive urine samples, 3 patients were positive for *L. pneumophila* in sputum samples by PCR, and 1 patient was positive by culture.

5.1. Conclusions

The detection of the soluble antigen of the *L. pneumophila* serogroup 1 in urine by an enzyme immunoassay (EIA) kit has proven as a rapid detection and more sensitive than PCR and culture for detection of *L. pneumophila* in respiratory specimens.

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Footnotes

Authors' Contribution: Mojtaba Moosavian: study concept and design, study supervision, data interpretation, critical revision and editing of the manuscript. Mahtab Khoshkholgh Sima: study concept and design, performing of the laboratory experiments, sample collection, data interpretation, statistical analysis and writing of the manuscript. Maryam Haddadzadeh Shoushtari and Seyed Mohammad Alavi: assistance in performance of this study. Mohammad Amin Fazeli Naserabad: assistance in sample collection. Effat Abbasi Montazeri: assistance in performing the PCR.

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