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Investigation of *Legionella pneumophila* and other *Legionella* species in atypical pneumonia patients

Atipik pnömonili hastalarda *Legionella pneumophila* ve diğer *Legionella* türlerinin araştırılması

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ABSTRACT

Objective: The aim of this study is to investigate *Legionella* species in 50 patients with atypical pneumonia, using culture, urinary antigen test and molecular techniques.

Methods: Non-selective BCYE-α media (Oxoid, England) and selective BMPA media (Oxoid, England) were used to isolate *Legionella* spp. from respiratory tract samples. The urinary samples of the patients were tested with the Alere BinaxNOW *Legionella* Urinary Antigen Card (Abbott, US) test to identify the presence of *L. pneumophila* serogroup 1 specific bacterial antigen. All respiratory tractsamples were tested with a commercial Duplicα RealTime *Legionella* pneumophila 23S rRNA specific region detection kit (Euroclone Diagnostica, Italy) and two home-made PCR methods. Home-made gel electrophoresis PCR tests were performed using Leg primers designed from 16S ribosomal RNA gene partial sequences for *Legionella* spp and primers targeting the Lmip (macrophage

ÖZET

Amaç: Bu çalışmada atipik pnömoni tanısı alan 50 hastada kültür, üriner antijen testi ve moleküler yöntemler kullanarak *Legionella* türlerinin araştırılması amaçlanmıştır.

Yöntem: Solunum yolu örneklerinden *Legionella* türlerinin izolasyonu için seçici olmayan BCYE-α (Oxoid, İngiltere) ve seçici BMPA (Oxoid, İngiltere) besiyerleri kullanılmıştır. İdrar örneklerinde *L. pneumophila* serogrup 1'e özgü bakteriyel antijenin varlığı Alere BinaxNOW *Legionella* Üriner Antijen Kart (Abbott, ABD) testi ile araştırılmıştır.Tüm solunum yolu örnekleri Duplicα RealTime *Legionella* pneumophila 23S rRNA spesifik bölgesini saptayan (Euroclone Diagnostica, İtalya) ticari kit ve iki laboratuvar yapımı PCR yöntemi ile test edilmiştir. Laboratuvar yapımı jel elektroforez PCR testinde *Legionella* spp. için 16S ribozomal RNA gen kısmi dizilerinden tasarlanan Leg primerleri ve *L. pneumophila* için Lmip (macrophage infectivity potentiator) genini hedefleyen primerler

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infectivity potentiator) gene for *L. pneumophila*. In the home-made real-time PCR test, primers targeting the lipopolysaccharide (lps) biosynthesis gene of *L. pneumophila* serogroup-1, the *L. pneumophila* mip gene, and the Legionella spp DNA region encoded by the 16S ribosomal RNA gene were used.

Results: The commercial Real-time PCR assay identifed the sequence of *L. pneumophila* 23S rRNA gene specific region in seven respiratory tract samples. Five samples were detected as Legionella spp. in home-made gel electrophoresis-based PCR and home-made Real-time PCR assay. Hovewer, all samples tested negative in the urinary antigen card test for *L. pneumophila* serogroup 1.

Conclusion: We conclude that the PCR positivities with three different molecular methods indicate that *Legionella* species other than *L. pneumophila* serogroup 1 should be investigated in the patients with atypical pneumonia using molecular methods. Also, our study demonstrates the significance of PCR methods in the investigation of Legionella species in clinical samples taken from patients with negative test results for *L. pneumophila* serogroup 1 specific urinary antigen test, but who are clinically considered to have *Legionella* pneumoniae.

Key Words: Legionella, *L. pneumophila*, Polymerase Chain Reaction (PCR), urine antigen, atypical pneumonia

kullanılmıştır. Laboratuvar yapımı Real-time PCR testinde ise, 16S ribozomal RNA geni tarafından kodlanan Legionella spp. DNA bölgesini, *L. pneumophila* mip genini ve *L. pneumophila* serogrup 1'in lipopolisakkarit (lps) biyosentez genini hedefleyen primerler kullanılmıştır.

Bulgular: Solunum yolu örneklerinden yedisinde ticari Real-time PCR testi ile *L. pneumophila* 23S rRNA genine spesifik bölge saptanmıştır. Bu örneklerden beşi Laboratuvar yapımı jel elektroforez tabanlı PCR ve laboratuvar yapımı Real-time PCR testleri ile Legionella spp. olarak tanımlanmıştır. Bununla birlikte, *L. pneumophila* serogrup 1 için üriner antijen testi tüm örneklerde negatif bulunmuştur.

Sonuç: Sonuç olarak; üç farklı moleküler yöntemle saptanan PCR pozitiflikleri, atipik pnömoni hastalarında *L. pneumophila* serogrup 1 dışındaki Legionella türlerinin de araştırılması gerektiğini düşündürmektedir. Çalışmamız özellikle *L. pneumophila* serogrup 1'e spesifik üriner antijen test negatifliği saptanan ancak klinik olarak *Legionella* pnömonisi olduğu düşünülen hastalardan alınan klinik örneklerin PCR yöntemi ile de araştırılmasının önemli olduğunu göstermektedir.

Anahtar Kelimeler: Legionella, *L. pneumophila*, Polimeraz Zincir Reaksiyonu (PZR), üriner antijen, atipik pnömoni

INTRODUCTION

Legionnaires' disease results from infections caused by the members of the *Legionellaceae* family. The *Legionellaceae* family includes at least 60 species and among these, *L. pneumophila* serogroup 1 is the most common pathogen. Responsible for approximately 90% of all *Legionella* infections (1,2).

Legionellaceae are commonly found in external environments due to their acid resistance and ability to survive in natural/artificial water systems and at high temperatures up to 66°C. These bacteria are transmitted to humans through the aerosolization of contaminated water (3).

Legionella infections are classified as nosocomial, community-acquired, and travel-acquired infections

(4). The cases can present sporadically or as outbreaks. Furthermore, they can cause hospital-acquired infections due to the bacterial contamination of hospital water systems or equipment (1,3,5).

The clinical picture associated with this microorganism can range from Pontiac fever associated with mild flu-like symptoms to severe pneumonia, also known as Legionnaires' disease. This bacterium can be fatal for the immunocompromised and individuals with underlying diseases or if left untreated (3,5).

Microbiological laboratory tests are crucial for differential diagnosis since it is almost impossible to differentiate Legionnaires' disease from other types of pneumonia solely through clinical and radiological examinations. Some of the genotypic and phenotypic examination methods used for diagnosis include bacterial culture, direct immunofluorescent antibody (DFA), urinary antigen, and serological and molecular tests (3,6,7).

This study aims to investigate the presence of *Legionella* species using bacterial culture, polymerase chain reaction (PCR), and urinary antigen methods in patients who were diagnoised with atypic pneumonia at chest diseases clinic of a tertiary hospital in Ankara, Turkey.

MATERIAL and METHOD

We investigated the presence of *Legionella* species in the respiratory tract and urine samples of 50 patients with atypical pneumonia diagnosis [35 males (70%) and 15 females (30%)] that had been treatedat the Chest Diseases Clinic between October 2014 and January 2016. The patients involved in the study, besides having had pneumoniae, additioanally had one or several of the following clinical or laboratory features: CNS abnormalities (irritation, mental confusion, stupor, lethargy, coma); cardiac abnormalities (relative bradycardia); gastrointestinal manifestations (abdominal pain, diarrhea); hepatic involvement (early or mild transient elevations of the serum transaminases); and/or electrolyte abnormalities (hypophosphataemia, hyponatraemia).

Bacterial isolation

In order to isolate *Legionella* species from patients' respiratory tract samples, we used the standard non-selective BCYE-α medium (ACES Buffer/ Potasyum hidroksid 5,0 g/500 ml; Ferrik pirofosfat 0,125 g/500 ml; L-sistein HCl 0,20 g/500 ml; α-ketoglutarat 0,50 g/500 ml) and the *Legionella*-selective BMPA medium [polymyxin B (8000 IU), anisomycin (8.0 mg), cefamandole (400mcg)] together. 49 sputum and one bronchoalveolar lavage fluid (BAL) samples were examined with gram staining and inoculated in 5% sheep blood agar, EMB, chocolate agar, BCYE-α (Oxoid, UK) and BMPA (Oxoid, UK) media. Approximately 1 mL of sputum was decontaminated using an HCl-KCl acid solution (pH 2.2) at a 1:10 dilution of the samples for 4 minutes and then re-cultured onto BCYE-α and BMPA media. The BAL fluid was not treated for decontamination. After the inoculation of the samples onto the media, they were incubated in a humid atmosphere at 36.5°C.

The bacteria growth in the media, starting from the third day, was monitored at 24 hours of intervals for fourteen days. Since *Legionella* species cannot reproduce on cysteine-free media, the colonies that grew on both selective media and sheep blood agar were not included in the assessment.

Real-time PCR, *Legionella pneumophila* 23S rRNA specific region

Nucleic acid isolation from clinical samples was performed using the manufacturer's instructions using the QIAamp DNA Mini Kit (Qiagen, Germany). We used the spin column method for this purpose. We used specific solutions (AL buffer, proteinase K, ethanol, wash buffer AW-1, wash buffer AW-2) for breaking down the bacterial cell wall, dissolving the DNA-protein complex, and separating it from other molecules, and centrifuge.

Following the extraction, the bacterial DNA was amplified and detected with the RotorGene 6000

(Corbett Research, Sydney, Australia) device and DuplicαRealTime *Legionella pneumophila* detection kit (Euroclone Diagnostica, Italy). The mixture with a total volume of 21 µL, including the amplification mixture, oligo mix, and internal control, was placed into 250 µL optical tubes as calculated per the procedure. Subsequently, isolated patient samples was added to obtain a total volume of 25 µL. Reaction conditions included 5 minutes of denaturation at 95 °C, followed by 45 cycles at 95 °C for 15 seconds and at 60 °C for 60 seconds.

In the studies carried out by the National Reference Laboratory for Respiratory Pathogens in Public Health Institution of Turkey, bacterial DNA was isolated from clinical specimens using the Biospeedy Nucleic Acid Isolation Kit (Bioeksen, Turkey). The presence of *Legionella* specific genomic structures in DNA isolates was investigated using standard PCR (home-made gel electrophoresis-based PCR) and home-made Real-time PCR methods.

Home-made gel electrophoresis-based PCR

Leg primers designed from the partial sequences of 16S ribosomal RNA gene for *Legionella* species, and the primers targeting the macrophage infectivity potentiator (L*mip*) gen for *L. pneumophila* were used as primers in home-made gel electrophoresis-based PCR assay (8-11).

Leg 448 A_F: 5'-GAG GGT TGA TAG GTT AAG AGC-3' and Leg 854 B_R: 5'-CGG TCA ACT TAT CGC GTT TGC T-3' primers were used for *Legionella* species16S rRNA gene. Furthermore, L*mip* L920_F: 5'-GCT ACA GAC AAG GAT AAG TTG-3' and L*mip* R1548_R: 5'-GTT TTG TAT GAC TTT AAT TCA-3' primers were performed for *L. pneumophila* macrophage infectivity potentiator (*mip*) gene.

For standard PCR, extracted DNA (5 µl each) were added to microtubes containing 20 µl of PCR mixture to obtain a total of 25 microlitres. The PCR mixture was prepared by using 2.5 µl of 10X PCR buffer, 2 µl of 25 mM MgCl2, 0.5 µl of 2.5 mMdNTP (200µM), 0.13µl each of Leg 448 and Leg 854 primers (50pmol/µl), 0.13 µl ofTaq DNA polymerase (5U/µl), 14.6 µl of molecular grade water. The preparations in the microtubes were initially denatured at 95°C for 5 minutes and then subjected to 40 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 45 seconds, and extension at 72°C for 1 minute using a thermal cycler (Bio-Rad T100). Then the final extension was performed at 72°C for 10 minutes. The PCR, containing L*mip* L920 and L*mip* R1548 primers, was performed with an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 45 seconds and followed by a final extension at 72°C for 1 minute (Bio-Rad T100). PCR-amplified DNA fragments were separated in 1.5% agarose gels with TBE buffer and visualized by ethidium bromide staining. A 100-bp DNA ladder was also used as a DNA size marker. *Legionella pneumophila* ATCC 43111 and *E. coli* ATCC25922 were used as positive and negative control strains in standard PCR tests.

Home-made Real-time PCR

The primers targeting the *Legionella* spp. DNA region encoded by 16S ribosomal RNA gene, mip gene of *L. pneumophila*, and lipopolysaccharide (lps) biosynthesis gene of *L. pneumophila* serogroup 1 were applied in home-made Real-time PCR assays. The primers and probes applied in home-made Real-time PCR assays were designed by National Reference Laboratory using NCBI database (unpublished data). The bacterial DNA was amplified and detected with home-made Real-time PCR in Bio-Rad CFX96 Real-Time System (Paris, France). The Real-time PCR mixture with a total volume of 8 µl, containing 100 µM stock primer & probe, 10 mM dATP, 10 mM dGTP, 10 mMd CTP, 10 mM dTTP, 25 mM MgSO4, 10x Reaction Buffer (KCl, Tris-HCl, pH 9.0, Triton X-100), 5 U/µl recombinant DNA polymerase and storage buffer were placed into 100 µl strip tubes. Subsequently, extracted DNA of 2 µl was added to obtain a total volume of 10 µl. The reaction conditions included 3 minutes of denaturation at 95 °C, followed by 39 cycles at 95 °C for 15 seconds and at 55 °C for 50

seconds.

Urinary Antigen Card Test

The immunochromatographic membrane-based assay was used to investigate the presence of *L. pneumophila* serogroup 1 antigens in patients' urine samples. The Alere BinaxNOW *Legionella* Urinary Antigen Card (Abbott, US) test was used in accordance with the manufacturer's instructions.

The study was approved by the Ankara Yıldırım Beyazıt Üniversity Clinic Research Ethics Committee (Date: 15.10.2014 and Number: 26379996/168).

RESULTS

The presence of *Legionella* species was investigated in the respiratory tract (49 sputa and 1 BAL fluid) and urine samples of 50 patients who were being monitored for atypical pneumonia. There were 27 inpatient (54%) and 23 outpatient (46%) subjects. According to medical histories of the patients, there were 1 (2%) hospital-acquired, 12 (24%) travel-acquired, and 37 (74%) community-acquired infections. There were 15 female and 35 male subjects, and the mean age was 56.3 years (range 21-87 years). Twenty-one (42%) patients were smokers, and four patients (8%) used alcohol. The subjects' comorbidities were as follows: coronary artery disease, 15 (30%); COPD (Chronic obstructive pulmonary disease) 7 (14%); and diabetes mellutis 5 (12%). The clinical, radiological, and laboratory findings of the patients are presented in Table 1. The most common symptom was cough and sputum (92%), and the most common radiological finding was unilateral lung infiltrate (54%).

We picked suspect colonies on specific BCYE-α and BMPA media and subcultured the colonies on BCYE-α and blood media and determined various Gr (+) and Gr (-) bacteria belonging to the throat flora. Culture testing did not reveal the presence of any specific *Legionella* species. All subjects tested negative in the urinary antigen card test for *L. pneumophila* serogroup1.

Duplicα RealTime *L. pneumophila* detection Real-time PCR assay identifed the sequence of *L. pneumophila* 23S rRNA gene specific region in seven (samples no; 13, 18, 24, 25, 31, 33 and 48) respiratory tract samples. The sociodemographic, clinical, and laboratory findings of patients who tested positive in Real-time PCR assay are summarized in Table 2.

Furthermore, as shown at Table 3, five of seven samples were detected as *Legionella* spp. in home-made gel electrophoresis-based PCR and home-made Real-time PCR assays.

Where at all seven samples, the presence of *L. pneumophila* rRNA was found by Real-time PCR method (Table 3).

Table 1. Symptoms and findings of all patients

Characteristics	Number (n)	(%)**
Symptoms*		
Fever	27	54
Cough	46	92
Sputum	46	92
Chest pain	28	56
Dyspnea	35	70
Hemoptysis	7	14
Diarrhea	12	24

Table 1 (cont.). Symptoms and findings of all patients

Characteristics	Number (n)	(%)**
Abdominal pain	11	22
Irritation	12	24
Confusion	2	4
Stupor	0	0
Lethargy	0	0
Coma	0	0
Radiography Findings		
Unilateral infiltrate	27	54
Bilateral infiltrate	11	22
Lobar infiltrate	4	8
Pleural effusion	2	4
Pulmonary cavitation	0	0
Patients without radiography results	7	14
Laboratory findings		
Elevated transaminases	11	22
Hyponatremia	9	18
Hypophosphataemia	8	16
Patients without laboratory findings	30	60

* Multiple parameters may be selected
** Indicates row percentages

Table 2. Findings of patients that tested Real-time PCR, *Legionella pneumophila* 23S rRNA specific region

Case	Age/Gender	Smoking Status	Alcohol Use	Chronic Disease	Symptoms	Laboratory Findings	Lung Radiography Findings
13	58/F	No	No	Hypothyroidism, Rheumatoid arth.	Cough, fever, dyspnea, sputum, chest pain	Normal	Normal
18	55/M	No	No	coronary artery disease	Cough, fever, dyspnea, sputum	Normal	Normal
24	32/F	Yes	No	Hypothyroidism	Cough, dyspnea, fever, chest pain	Normal	Unilateral infiltrate
25	32/F	No	No	Asthma	Cough, fever, dyspnea, sputum, chest pain	Normal	Unilateral infiltrate
31	52/M	Yes	No	Asthma	Cough, fever, dyspnea, sputum, chest pain	Elevated transaminases, hyponatremia	Unilateral infiltrate
33	45/M	Yes	No	No	Cough, fever, dyspnea, sputum, chest pain	Elevated transaminases	Unilateral infiltrate
48	75/M	No	No	Asthma, Liver disease	Cough, dyspnea, fever sputum, irritation	Normal	Unilateral infiltrate

Table 3. Comparison of PCR results

Sample No	Real-time PCR*	Home-made gel electrophoresis-based PCR		Home-made Real-time PCR		
	L. pneumophila 23S rRNA	*Legionella spp (Leg gene)*	*L. pneumophila (Lmip gene)*	*Legionella* spp (16S rRNA)	*L. pneumophila (mip* gene)	*L. pneumophila* serogroup 1 (*lps* gene)
13	**Positive**	**Positive**	Negative	**Positive** (Cq** 25,30)	Negative	Negative
18	**Positive**	**Positive**	Negative	**Positive** (Cq 33,30)	Negative	Negative
24	**Positive**	**Positive**	Negative	**Positive** (Cq 26,27)	Negative	Negative
25	**Positive**	**Positive**	Negative	**Positive** (Cq 30,27)	Negative	Negative
31	**Positive**	Negative	Negative	Negative	Negative	Negative
33	**Positive**	Negative	Negative	Negative	Negative	Negative
48	**Positive**	**Positive**	Negative	**Positive** (Cq 25,24)	Negative	Negative

***DuplicαRealTime *Legionella pneumophila* Detection kit (Euroclone Diagnostica, Italy)**
****Cq: Cycle**

DISCUSSION

It is impossible to differentiate Legionnaires' disease from other types of pneumonia solely through clinical and radiological examinations. Therefore, genotypic and phenotypic testing methods are prominent in determining the causative microorganism (6). The methods that are used to detect *Legionella* species all have various advantages and disadvantages of their own. For instance, some disadvantages include that the culture method is time-consuming and has a low sensitivity, the urinary antigen test can only detect *L. pneumophila* serogroup 1, and PCR targets different gene regions (1,7). PCR is a diagnostic method that is more sensitive than the bacterial culture in the differentiation of Legionnaires' disease. That being said, according to the standard Case Definition of Legionnaires' Disease, a clinically compatible case tested positive in PCR indicates "probable" Legionnaires' disease (8). Therefore, a patient that tests positive in PCR needs to be confirmed by sequence analysis or another method. Researchers can investigate various bacterial DNA regions, including the *Legionella mip*, 5SrRNA, and 16S rRNA regions, and the 23S-5S rRNAintergenic spacer region for this purpose (1,3,8). Using bacterial cultures as a reference, Cloud et al. found the sensitivity of PCR to be 100% for the 16S rRNA gene region of *Legionella* species (12). Nazarian et al. investigated the validity of the determination of *Legionella* spp. 23S rRNA with PCR assay and reported the specificity to be 100% and the sensitivity to be 100% for clinical samples and 98.6% for environmental samples (13). In our study, we amplified the *L. pneumophila* 23S rRNA gene specific region, which can detect all serogroups of *L. pneumophila* and found that seven patients tested positive.

Five of these samples tested positive for *Legionella* spp. 16S rRNA in the home-made real-time PCR assay and for *Legionella* spp. Leg region in the home-made gel electrophoresis-based PCR assay. The home-made real-time PCR assays targeting *L. pneumophila* and

L. pneumophila serogroup-1 and the home-made gel electrophoresis-based PCR assays that targeted the *L. pneumophila mip* gene region were not positive for any subject.

It is known that the sensitivity and specificity of PCR methods in diagnosing *Legionella* infections vary according to the targeted genomic regions. Besides, in this study, the clinical samples were tested by Real-time PCR for *L. pneumophila* 23S rRNA specific region just after the sampling was made. However, clinical samples had been stored at −20°C (deep freeze) until home-made Real-time PCR and home-made gel electrophoresis-based PCR methods were carried out. Moreover, bacterial DNA was isolated from clinical specimens using the different Bacterial DNA Isolation Kits, such as QIAamp DNA Mini Kit (Qiagen, Germany) and Biospeedy Nucleic Acid Isolation Kit (Bioeksen, Turkey) in the PCR methods studied. Hence, we believe that all these affect the sensitivities of the PCR tests used in the study and finally, the results of our study.

The bacterial culture method is accepted as the gold standard for the diagnosis of Legionnaires' disease (1,3,7). Indeed, the standard Case Definition of Legionnaires' Disease evaluates the isolation of the bacteria from patient samples in a clinically compatible case as "confirmed" Legionnaires' disease. The sensitivity of the method depends on factors such as the total number of bacteria in the specimen, the vitality of the bacteria, the stage of infection, antibacterial drug use, and the quality of the respiratory tract specimen (7). In our study, we were unable to isolate any bacteria from the *Legionella*cea family.

Divan Khosroshahi et al. reported that 12% of the respiratory tract samples of 109 patients suspected of ventilator-associated pneumonia tested positive in PCR for *L. pneumophila* but were negative in bacterial cultures ascribed to the bacterial airway load and the vitality of the bacteria (1). Similarly, authorsof another study concluded that while PCR results for the two gene regions (*L. pneumophila* mip gene and *Legionella* genus 16S rRNA gene) were completely correlated, bacterial culture was inadequate in detecting Legionnaires' disease (14). Chen et al. studied a large number of samples and determined the sensitivity of culture, Real-time PCR assay targeting the mip gene for *L. pneumophila*, and urinary antigen tests to be 50%, 92%, and 96%, respectively (15).

Urinary antigen tests are preferred for their ease of use in clinical diagnosis, but their most significant disadvantage is that they can only detect the cell-wall antigens of *L. pneumophila* serogroup-1. Hovewer, *L. pneumophila* serogroups 2-16 and other *Legionella* species (*L. bozemanii, L. micdadei, L. longbeach, L. dumoffii, L. anisa,* etc.) can also cause *Legionella* infections (2).The urinary antigen test's other disadvantages include potential false positivity due to presence of rheumatoid factors and the cross-reactions between other *L. pneumophila* serogroups and other *Legionella* species (16). We believe that the reason why the patients that were tested positive in PCR, tested negative in the urinary antigen test might be due to the fact that the urinary antigen test can only detect *L. pneumophila* serogroup 1. Our interpretation of this result was that non-*L. pneumophila* serogroup 1 species were more prevalent than *L. pneumophila* serogroup 1 in our region.

On the other hand, Mojtahedi et al. found urinary antigen test positivity in 16.7% and *L. pneumophila* 16S rRNA gene PCR positivity in 19.8% of their patients. The authors' concluded that PCR is a better detection method for *Legionella* infection than urinary antigen test (17).

The clinical picture associated with this microorganism can range from mild flu-like symptoms, called Pontiac fever, to severe pneumonia, also known as Legionnaires' disease. In our study, patients tested positive in PCR all had cough, fever and dyspnea. These were followed by sputum and chest pain. We determined the underlying risk factors from most common to least as asthma, COPD and smoking.

When the laboratory findings were evaluated, it was observed that one patient had elevated transaminases and hyponatremia, and one patient had only elevated transaminases. Five of the PCR-positive patients had unilateral infiltrate on chest X-ray. Similar to our study, Erdoğan et al. reported fever in all patients diagnosed with Legionnaires' disease and elevated transaminases in two patients. Contrary to our results, they determined smoking as the primary risk factor for patients (18). The underlying risk factors, symptoms, and laboratory findings of our subjects are compatible with other studies (19-22).

We conclude that the PCR positivities with three different molecular methods indicate that *Legionella* species is a considerable pathogen of patients with atypical pneumonia. Physicians should consider *Legionella* species to increase the effectiveness of the treatment and reduce mortality, particularly during the peak periods of the disease. Also, our results indicate that urinary antigen test negativity should not exclude other species and serogroups. For this purpose, the patients should be investigated for serogroups other than *L. pneumophila* serogroup 1 using a second method such as PCR. Our study demonstrates the significance of PCR in the investigation of *Legionella* species and serogroups. Furthermore, PCR has been shown to have sufficient sensitivity to detect the pathogen, and after extensive studies, maybe an alternative for other test methods. The clinician and the microbiology laboratory should be cooperating quickly, accurately, effectively, and reliably in diagnosing the patient, initiating a specific treatment and taking effective control measures.

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ETHICS COMITTEE APPROVAL

* The study was approved by the Yıldırım Beyazıt University Clinic Research Ethics Committee (Date: 15.10.2014 and Number: 26379996/168).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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