

**Original article**

**Isolation and identification of legionellosis agents from fishponds, swimming pools and cooling towers in Khuzestan province, Iran**

**Mojtaba Moosavian, PhD  
Amir Dashti, MSc**

Department of Medical  
Microbiology, School of Medicine,  
Infectious and Tropical Diseases  
Research Center, Ahvaz  
Jundishapur University of Medical  
Sciences, Ahvaz, Iran

**Address for correspondence:**

Dr. Mojtaba Moosavian,  
Department of Medical  
Microbiology, School of Medicine,  
Ahvaz Jundishapur University of  
Medical Sciences, Ahvaz, Iran  
Tel: +98611 3330074  
Fax: +98611 3332036  
Email: moosavian\_m@yahoo.com

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**Abstract**

**Introduction and objective:** *Legionella* are the causative agents of pneumonia in human and it is reported that up to 90% cases of legionnaires` disease are due to *Legionella pneumophila*. These organisms are ubiquitous distributed in natural and man made water sources. They are spread to human by inhalation or aspiration of contaminated aerosols of these sources. We studied some of man made water sources in view of the presence of *Legionella*, by two methods of culture and PCR.

**Materials and methods:** One hundred and fifty water samples collected from different man made water sources were examined. After acidic treatment of samples, water pellet was inoculated onto buffered charcoal yeast extract agar (BCYE) and BMPA (BCYE supplemented with three antibiotics) media. Isolated colonies were identified by morphological and biochemical tests. DNA was extracted from the bacteria and was used for PCR technique. DNA pattern of *Legionella* were identified after electrophoresis of PCR products.

**Results:** Survey of water samples collected from different sources resulted in isolation of *Legionella pneumophila* (7.3%) by culture, and identification of them (15.3%) by PCR. The highest isolates of *L. pneumophila* were from fish ponds in rates of 6.6% by culture and 13.3% by PCR method. Sensitivity and specificity of PCR in this survey were 100% and 95%, respectively.

**Conclusion:** This study showed that legionnaires` disease agents were widely spread in our examined water sources and compared with culture; PCR method has suitable sensitivity and specificity for rapid detection of these organisms in environmental water sources.

**Significance and impact of the study:** The results of this study will increase physicians and microbiologists awareness about spreading of *Legionella* and this will be useful for control of legionellosis agents.

**Keywords:** *Legionella*; Legionnaires` disease; Fishponds; Swimming pools

## Introduction

*Legionella pneumophila* (LP) is a Gram-negative bacillus that causes nosocomial and community-acquired pneumonia [1,2]. This species accompanies more than 50 other species of *Legionella*. These bacteria are ubiquitous in water sources and are transmitted by inhalation or aspiration of contaminated aqua's aerosols [3]. The most outbreaks of Legionnaires' Disease (LD) are linked to contaminated water system such as cooling towers, air conditioning system, water distribution devices, potable hot water systems, fishponds, spa water, and equipment of respiratory treatments [2,4-8].

An important strategy in the prevention of legionellosis is routine culture of the hospital water supply [8]. Culture is gold standard for detection of *Legionella* species and if the specimen is appropriate, it will be a definitive method for diagnosis [9,10]. The sensitivity of culture is 50 to 90% [10] and its specificity is presumed to be 100% [3]. However, a special medium is required [11] and the organisms take three to five days to form visible colonies. Direct fluorescent-antibody (DFA) test was the first method devised for direct examination of *Legionella* in the specimens, however, the sensitivity of this test is low (25-75%) and  $10^4$  to  $10^5$  bacteria should be present for the test to become positive [12].

A polymerase-chain reaction (PCR) was designed for improving the speed, accuracy and sensitivity of diagnosis of *Legionella* [12,13]. PCR represents one of the few diagnostic tests with the potential to detect infections caused by all of the known species of *Legionella* [13,14]. Various PCR tests have been reported for *Legionella* target such as the 5S rRNA gene or the mip gene. This method was developed by using the 16S rRNA target gene, which exists in multiple copies per genome and was indicated to be highly sensitive and specific

[14,15]. The aim of this study was to identify *Legionella* in different water sources such as fishponds and swimming pools in addition to cooling towers by culture and PCR methods.

## Materials and methods

### Samples

One hundred and fifty water samples collected accidentally from fishponds, swimming pools and cooling towers, were examined. The fishponds were located in some cities of Khuzestan province, Iran, (Such as, Baiooz, Sheiban, Mollasani, Shooshtar and Abadan). Also swimming pools were located in Ahvaz (the center of Khuzestan province), but the places of cooling towers were in faculties or hospitals of Ahvaz Jundishapur University of Medical Sciences, Iran.

### Acid treatments

The collected water samples (each one 500ml), in sterile bottles were transported to the microbiology lab in the school of medicine. After centrifugation in 2500rpm for 15mins, the supernatant was removed from each sample and 0.5ml of the pellet was transferred to 4.5ml HCl-KCl acidic buffer (pH 2.2). The treated samples were inoculated on buffered charcoal yeast extract agar (BCYE-non selective medium, Oxoid Ltd. UK) and BMPA (BCYE supplemented with three antibiotics-selective medium, Oxoid Ltd. UK) and the plates were incubated in candle jar (3-5% CO<sub>2</sub>) at 37°C and about 90% humidity for up to six days.

The suspected colonies to *Legionella* were identified by Gram staining, growth on BCYE, but not on sheep blood agar (Merck, Germany) and Eosin Methylene Blue Agar (EMB, Merck, Germany). In addition, isolated *Legionella* were detected more by biochemical tests such as catalase,

oxidase, gelatinase, beta-lactamase tests and hydrolysis of hippurite [16].

#### DNA extraction

A portion of the same pellet was used for DNA extraction based on manufacturer direction of the kit (CinnaGen Inc. Iran). In this study *L. pneumophila* and distilled water were used as positive and negative control, respectively.

#### DNA amplification

The extracted DNA was amplified in a portion of the 16S rRNA gene by using the forward primer JFP-5`-AGGGTTGATAG-GTTAAGAGC-3` and Reverse primer JRP-5`CCAACAGCTAGTTGACATCG-3` [15] in a thermocycler (Techne Co. UK). Master mixture was prepared in each 25µl reaction including 2.5µl in 10X PCR buffer, 2.5µl of dNTP mix (2mM), 1.5µl MgCl<sub>2</sub> (25mM), 2.5µl of forward and reverse primers (10µM), 1.25µl of Taq DNA polymerase (0.625U/25µl), 5µl of DNA template and 9.5µl of distilled water. Initial denaturation at 93°C for 45s was followed by 30 cycles of annealing at 55°C for 30s, extension at 72°C for 90s and a final step of extension at 72°C for 5mins. The PCR product (7.2µl) was mixed with 1.8µl of loading buffer and electrophoresed on 1.5% agarose gel in 1X TBE buffer (5mM Tris, 5mM boric acid, 0.1mM EDTA) at 120V for 90mins. The DNA fragment was visualized using ethidium bromide staining.

#### Results

Based on the obtained results, we identified 11 isolates (7.3%) of *L. pneumophila* by culture and 23 isolates (15.3%) of the same species by PCR (Table1). Out of 20 water

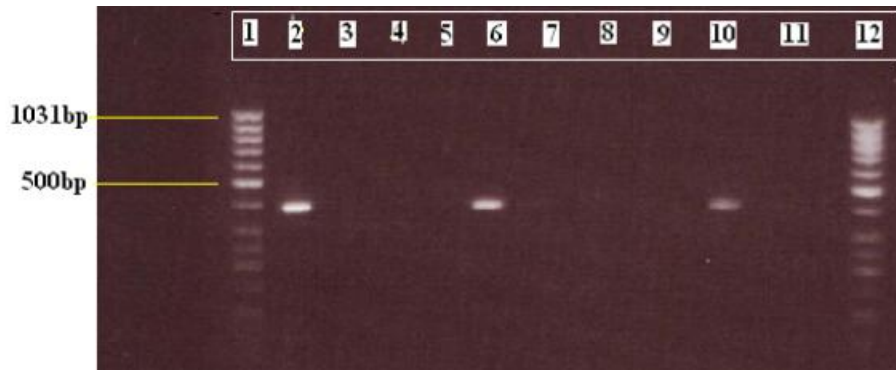
samples collected from 10 swimming pools in different sites, one strain of *L. pneumophila* (5%) was detected by PCR, but the culture results of these samples were negative. Sensitivity of PCR in this study was 100%, but its specificity was 95% for swimming pools and 92% for cooling towers. One hundred and seventeen samples out of 150 examined samples were taken from fishponds in five points of Khuzestan province. Ten strains of *L. pneumophila* (8.6%) were isolated from them by culture, while PCR could detect 20 strains of these species (17.1%) in the same water sources (Table 2 and Fig. 1).

**Table 1:** Examined water sources and number of *L. pneumophila* isolates which identified by culture and PCR

Water sources	Total	Culture		PCR	
		No.	%	No.	%
Fish ponds	117	10	6.6	20	13.3
Swimming pools	20	0	0	1	0.7
Cooling towers	13	1	0.7	2	1.3
Total	150	11	7.3	23	15.3

**Table 2:** Frequency of examined water samples and *L. pneumophila* isolated from fish ponds in cities of Khuzestan by culture and PCR

Fish ponds sites	Total		Culture		PCR	
	No	%	No	%	No	%
Shooshtar	50	42.7	3	2.6	7	6
Abadan	27	23.1	2	1.7	2	1.7
Baiooz	20	17.1	5	4.3	10	8.5
Sheiban	12	10.3	0	0	0	0
Mollasani	8	6.8	0	0	1	0.9
Total	117	100	10	8.6	20	17.1



**Fig. 1:** Agarose gel electrophoresis of PCR products of *L. pneumophila* isolated from fishponds. Lanes 1 & 12 are molecular size marker. Lane 2 is positive control. Lane 3 is negative control. Lanes 6 & 10 are positive samples (*L. pneumophila*). Lanes 3-5, 7-9 and 11 are negative samples

The results of this study also showed only one strain of *L. pneumophila* (7.7%) to be isolated by culture from 13 samples collected from cooling towers, while two strains of this bacterium (15.4%) were identified in these sources by PCR (Table 3).

**Table 3:** Frequency of examined water samples and *L. pneumophila* isolated from cooling towers in central city of Khuzestan by culture and PCR

Cooling tower sites	Total		Culture		PCR	
	No	%	No	%	No	%
Faculty of Medicine	2	15.4	0	0.0	0	0.0
Faculty of Dental	3	23.1	0	0.0	0	0.0
Faculty of Pharmacy	1	7.7	0	0.0	0	0.0
Shafa Hospital	1	7.7	0	0.0	0	0.0
Golestan Hospital	6	46.1	1	7.7	2	15.4
<b>Total</b>	<b>13</b>	<b>100</b>	<b>1</b>	<b>7.7</b>	<b>2</b>	<b>15.4</b>

**Discussion**

*Legionella pneumophila* causes nosocomial and community-acquired pneumonia that can range from mild respiratory illness to acute life-threatening pneumonia [1,2]. The majority of LD is caused by *L. pneumophila*

serogroup1 [17-19]. However, diagnosis of LD can be difficult as clinical symptoms and some of laboratory methods which are based on both phenotypic and genotypic are not helpful in diagnosis [20].

In this study, frequency of *L. pneumophila* in 150 examined water samples was 7.3% by culture, while PCR could detect 15.3% of these bacteria. Statistical analysis showed a significant difference ( $P < 0.05$ ) between the results of PCR and culture of water samples. However, the results which obtained from culture and PCR were different for each of three man-made environment water sources. The culture results of 20 water samples which were collected from swimming pools showed no isolation of *Legionella* strain, while PCR of these samples could detect one strain (5%) of this bacterium.

The negative result of swimming pools water may be dependent to chlorination and remainder of chlorine gas in the pools water. Some of the studies also indicated that remaining more than 2mg/l of chlorine in water could eradicate *Legionella* [21]. Also, absence of *Legionella* growth effective-factors, such as the presence of other bacteria in pool water, warm water temperature, the presence of free-living protozoa and the nutritional factors [13,22] could be reasons of the absence of *Legionella* in these sources.

In spite of a few reports about isolation of *Legionella* from some environmental water sources in Iran [11,23] fishpond as a major water living environment has not yet been surveyed in our area for existence of *Legionella*. These water sources as well as swimming pools are two important places, which are used by many of peoples in warm-weather area of Khuzestan province, in south-west of Iran. Detection rate of LP in fishponds by PCR (13.3%) was two times of this rate by culture (6.6%) which showed a significant difference between PCR and culture results of these water sources ( $P < 0.05$ ).

Although the most number of examined water samples were belonged to fishponds of Shooshtar (#50), Abadan (#27) and Baiooz (#20), the numbers of *Legionella* strains which were detected by PCR in these sources were 7, 2 and 10, respectively (Table 2). Also, the relative frequency of LP obtained by culture was less than PCR in all of the above sources. By the way, no *Legionella* strain was detected in Sheiban fishpond by both culture and PCR methods, in spite of 12 examined samples from this source. In comparison with some studies, which showed the rate of *Legionella* in the fishponds by culture method to be 6.7% (two isolates from 30 ponds) [5] culture of the water samples in our study revealed more isolates of this bacterium. However, the result of the present study was the same as another's on freshwater environments which showed identification rate of *Legionella* as many as 80% (two times) by PCR and 40% by culture [9,24].

The biochemical tests in our study showed that the species of all *Legionella* isolates were pneumophila. These results were confirmed by PCR, using the special primers which were complimentary to 16s rRNA gene. PCR assay in this study could detect all *Legionella* culture-positive in water samples. Therefore, sensitivity of test

was 100%. The Culture-negative samples, which were PCR positive, were considered as false-positive results. So, specificity of our study was 95% and 92% for swimming pools and cooling towers, respectively. However, some studies indicated that the PCR detection of *Legionella* infections has a moderate sensitivity and a high specificity [13].

### Conclusion

We concluded that with due to the use of swimming pools and fishponds by many of people, especially in summer season, looking for *L. pneumophila* in such sources by PCR or at least culturing of water samples will indicate the rate of environment water colonization. In addition, it will draw the notice of physicians that to consider these sources as a suspicious cause of environment acquired LD.

**Conflict of interest statement:** All authors declare that they have no conflict of interest.

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