Detection of *Legionella pneumophila* and *Legionella dumoffii* Biochemically in Water Samples in Baghdad City, Iraq

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Abstract

Background: Legionellae can be isolated from various sources of water. *Legionella pneumophila* is transmitted via contaminated water and caused many diseases like pneumonia; lungs abscesses and fever.

Objectives: Isolation and identification of *L. pneumophila* from environmental samples; study the factors that affect the frequency of *L. pneumophila*; and some oxidizing substances on their growth.

Materials and Method: A total of one hundred water samples were collected from Cooling tower; Water tanks; Tap water and Swab from inner tap water. All samples were cultured on Buffered charcoal yeast extract (BCYE) agar and Buffered charcoal yeast extract (BCYE) broth to isolate *Legionella pneumophila* and *Legionella dumoffii*. Effect of pH; temperature; chlorine; Iron and effect of soluble zinc on *L. pneumophila* growth were studied.

Results: The isolates gave positive results for the tests of catalase, oxidase, gelatin degradation, nitrate reduction and beta-lactamase. Iron; Soluble Zinc; Chloride; Calcium hardness ($CaCO_3$); Turbidity and silver were ≤ 3ppmas; 1-3 ppmas; 0.2-0.5 ppm; 600 ppm; ≤ 0.3 and 3 ppmas respectively. The distribution of *L. pneumophila* in the water samples were studied.

Conclusion: Fatal*L. pneumophila* may be transmitted by drinking contaminated water and led to death because they caused pneumonia.

Keywords: *Legionella pneumophila, Legionelladumoffii, Water sources, Antibiotic resistance.*

Introduction

Legionella pneumophila (*L. pneumophila*) is a Gram negative bacillus, grow and multiply under aerobic conditions in the presence of cysteine and iron, hydrolyses gelatin and produces urease. It is positive for oxidase and catalase. The colonies colour is white to greyish. It grows on yeast extract agar[1]. It has been isolated from patients suffering from Legionnaisis (respiratory system infections), drinking water, lakes, hot water tanks and cooling towers. *L. pneumophila* isolated from tap water; and from tankers in Baghdad and Basra cities. Many Iraqi researchers found that 75 isolates of *L. pneumophila* were isolated from 96 water samples which susceptible to many antibiotics (Gentamicin, Streptomycin, Rifampin, chloramphenicol) and resistance to Penicillin and Cephalothin [2]. Another study revealed that one hundred ten*L. pneumophila* isolates were isolated from cooling towers in General Company for the manufacture of biofertilizer/Basra, (96.3%) of isolates were sensitive to Chloramphenicol and the Refampein while (14.5%) of isolates were sensitive to Polymaxin B [3]. Forty-nine isolates of *L. pneumophila* were isolated from 222 precipitation water tanks and filtration tanks in Basra governorate. All isolates were resistant to ampicillin while susceptible to doxycycline [4]. Most of Legionnaires' Diseases (90%) caused by *L. pneumophila*, followed by *L. dumoffii*[5]. The aims of the current study are isolation and identification of *L. pneumophila* from environmental samples; study the factors that affect the frequency of *L. pneumophila*; and some oxidizing substances on their growth.

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Materials and Method

1. **Water sample collection:** A total of one hundred water samples (1000 mL from each sample) were collected from:

- Cooling tower (25 samples).
- Water tanks (25 samples).
- Tap water (25 samples).
- A swab from inner tap water (25 samples).

Each sample was collected in a sterile glass container. All samples were transported to the laboratory immediately in the icebox.

2. **Water samples concentration[6,7,8]:** the concentrations of the water samples were done by centrifugation at 3000 round per minute for 5 minutes. The supernatants were discarded, and the sediments were kept in the sterile container for minutes. Then the sediments were concentrated by using Millipore membrane filter (with pore size 0.2 μm). The filter membrane resuspended with 10 mL of original water and vortexed for mixing (400 r.p.m. for 10 minutes). Then 0.1 mL of samples were cultured on *Legionella* isolation media (triplicate plates):

- Buffered charcoal yeast extract (BCYE) agar (Oxoid) containing (Yeast Extract 10 g/L; Charcoal 2.0g/L; Ferric Pyrophosphate 0.25 g/L; ACES Buffer 10.0g/L; Potassium Carbonate 2.3 g/L and Agar 14.0g/L) supplemented with a-ketoglutarate, vancomycin, polymyxin B, and anisomycin. The pH of medium adjusted to 6.9.
- Buffered charcoal yeast extract (BCYE) broth (Oxoid) to encourage the growth of *Legionella*. Then spread on BCYE agar.

All plates were incubated at 37C° with 2% CO_2. The culture media plates were examined after 2, 3, 5, 6, and 7 days of incubation to detect the *Legionella* colonies (white, convex, circular, 2 mm in diameter, like ground glass in their appearance. While the swabs of water samples were cultured onBCYE agar directly; the plates were incubated at 37C° with 2% CO_2 and then examined after 5 days of incubation.

3. ***L. pneumophila* identification:** Many biochemical tests were done to identify ***L. pneumophila*** [9]:

- Gram staining.
- Urease test.
- Nitrate test.
- Oxidase testing (using 1% *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine dihydrochloride.
- Catalase test (using Hydrogen peroxide; 3% H_2O_2).
- Gelatin liquefaction test (using gelatin agar stab).

4. **Effect of pHon *L. pneumophila* growth:** Tubes containing 10 mL of nutrient broth (Oxoid) with L-cysteine in various ranges of pH (6, 6.5, 7, 7.5, and 8) were inoculated with *L. pneumophila* isolates. All tubes after inoculation with bacteria under study were incubated at 37C° for 5 days. Then, the turbidity of each tube was measured by using turbidimeter apparatus to determine the growth of *L. pneumophila* in different pH values. After that, 0.1 mL of bacterial growth was taken from each tube and inoculated on BCYE agar; the plates were incubated at 30°C for 5 days to calculate the number of viable bacteria in different pH levels [10].

5. **Effect of chlorine on *L. pneumophila* growth:** It was donewith serial dilutions of free chlorine (0.1-1.5) mg/L in sterile 0.85% normal saline. All tubes after inoculation with bacteria; were incubated at 25°C for 30 minute. 0.01 ml from each dilution was inoculated on BCYE agar and incubated to study the effect chlorine on bacteria [11]

6. **Effect of temperatures on *L. pneumophila* growth:** All isolates were cultured on BCYE agar in incubated in different temperatures (20, 25, 30, 35, 37, 40 and 45) °C [11].

7. **Effect of soluble zinc on *L. pneumophila* growth**[12]

8. **Effect of Iron on *L. pneumophila* growth**[12]

9. **Turbidity measuring**[13]

Results and Discussion

The results showed that the isolated bacteria were Gram negative bacilli, motile, grown on (CYEA), which contains the yeast extract and cysteine. The isolates gave positive results for the tests of catalase, oxidase, gelatin degradation, nitrate reduction and beta-lactamase. They gave negative results to ferment sugars (glucose, maltose, lactose); and showed a bright blue color under ultraviolet light. So, the isolates were identified as the *Legionella pneumophila*. Analysis results of water samples were fixed in the table (1) and (2).

Table 1: Biochemical analysis results of water samples

Iron (Fe^{+2}) (ppmas)	Soluble Zinc (Zn^{+2}) (ppmas)	Chloride (ppm)	Calcium hardness ($CaCO_3$) (ppm)	Turbidity	Ag (ppmas)
≤ 3	1-3	0.2-0.5	600	≤ 0.3	3
ppmas: part per mas Ag: Silver ppm: part per million					

The distribution of*L. pneumophila* in the water samples were 22(34.37%); 18(28.12%); 15(23.43%) and 9(14.06%) in cooling tower; tanks; tab water and swab from tab water respectively. The frequency of isolates number 1 from positive samples were 22,15,14,9for cooling tower, water tanks, tab water and swabs from tab water respectively.

Table 2: Distribution of *Legionella pneumophila* isolates according to water sources

(%) Isolate of no. 2 from total positive sample	Frequency of isolate no. 2 from positive sample (%)	Isolate of no.1 from a total positive sample (%)	Frequency of isolate no.1 from positive sample	Negative sample (%)	Positive sample (%)	No. of sample	Source of samples	No
80(20/25)	20(46.51)	88(22/25* 100)	22(36.66)	3(9.33)	22(34.37)	25	Cooling tower	1
48(12/25)	12(27.91)	60(15/25* 100)	15(25)	7(19.44)	18(28.12)	25	Tanks	2
40(10/25)	10(23.25)	56(14/25* 100)	14(23.33)	10(27.77)	15(23.43)	25	Tab water	3
4(1/25)	1(2.32)	36(9/25* 100)	9(15)	16(44.44)	9(14.06)	25	Swab from tab	4
172172/4=43	**43(100)**	**240(240/4=60)**	**60(100)**	**36(100)**	**64(100)**	**100**		**Total**

In one positive sample; two isolates may appear and in some samples, only one isolate appeared (frequency).

Two species of *Legionella* were isolated from water samples. The first was(60) isolates of *Legionella pneumophila*; and the second was (43) of *L. dumoffi*. The frequency of two species of *Legionella* in the cooling tower was more than other water samples, and the frequency of *Legionella pneumophila* was more than the frequency of *L. dumoffi*.

The frequency of *L. pneumophila* at pH_7 (88.3%) and this was more at pH_8 (60, 61.1 %) respectively. Whereas, the frequency at temperature 37°C (83.3%) was more than at 40°C and 45°C (73.3 and 63.37)% respectively. The current results showed also that the frequency of *L. pneumophila* was better at 0.3 ppm of R. chlorine when compared with 0.5 and 0.7 ppm of R. chloride. The frequency of *L. pneumophila* was high at 10 ppm of dissolved Oxygen and 3 ppm of Iron. Ag (silver) inhibited the growth of *L. pneumophila* at a concentration of 3ppm (table 2).

The results of the current study were incompatible with other studies. In Iran, only one isolate (2.9%) of *L. pneumophila* was isolated from water [11]. Whereas, in Saudi Arabia;the rate of *L. pneumophila* in water tanks was (8%), and most the isolates were grown better in pH_6 and pH_7; survived in temperature at 42°C and normal level of chlorine in water tanks. The other researches also isolated *L.dumoffi* (2%)[12].

The results of the present study are compatible with Al-Sulami*et al* [4]. *L. pneumophila* isolates were detected in 6 from 19 water stations in Basra governorate; the average of residual chlorine concentration was (0-1.03) mg/L.

Legionella pneumophila was emphasized for frequency in all water samples collected and it was the predominant species among other species affiliated with the *Legionella* spp. The spread of this species is evidence of the favourable environmental conditions, which increased the chance their presence in the samples, were indicated that these bacteria survive in temperatures between 40-42 °C, pH between 5.6-8.7 and high concentrations of chlorine ranged from 5.0-0.6 µg/ml [13].

Felice *et al*[14] isolated *L. pneumophila* from water

pools in Venezia Giulia, Italy. The prevalence of *L. pneumophila* was (82% of positive samples). In the Netherlands, 33.2% of water drinking water samples taking from buildings had *L. pneumophila*. This study was done to re-plan the drinking water management because *L. pneumophila* considered a dangerous bacterium which causing pneumonia and urinary tract infections [15]. 89 samples (43.6%) of 204 water samples (showerhead, taps in kitchens and tanks) had *L. pneumophila* in Kuwait, diagnosed with Real-Time Polymerase Chain Reaction[16]. Other studies showed that the prevalence of *L. pneumophila* in different water sources was (80%) in Mosul governorate/North of Iraq. The isolated *L. pneumophila* isolates were killed at 55°C for 30 min and 70°C for 5 min. Also, the isolates of bacteria were killed after exposure to UV light; 70% ethanol; 20% Isopropanol and 1% formalin [17]. Gauad*et al*. [18] determined the frequency of *L. pneumophila* in patients suffered from pneumonia and urinary tract infections using PCR technique. The percentage of *L. pneumophila* was 30% at hospitals in Baghdad city. The researchers suspected that the sources of *Legionella pneumophila* were drinking water or ventilation and cooling opening [18]. In Australia, the sources of *L. pneumophila* are shows, washing machines, swimming pools and lakes. The most isolates of *L. pneumophila* isolated from the water with temperature ranged from (>20 °C) to (<60 °C), and water containing free residual chlorine (<0.5 mg/L) [19-22].

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

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